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Immunological Localization of Plant Secondary Metabolites

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A Thesis

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of

Biology

Presented in Partial Fulfillment of the Requirements
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October 1989

ABSTRACT

Immunological Localization of Plant Secondary Metabolites

Louise Brisson, Ph.D.
Concordia University, 1989

Plants are known to accumulate a variety of organic substances which seem to play no conspicuous role in plant growth and development, although several reports have documented the ecological significance of these secondary metabolites. However, very little is known about their site of accumulation at the tissue, cellular and subcellular levels. To address this problem, polyclonal antibodies were used for the localization, by immunofluorescence and immunocytochemistry, of Chrysosplenium flavonoids and Catharanthus alkaloids. Localization of these natural products was achieved with an indirect method: rabbit antibodies against the secondary metabolite followed by goat antibodies against rabbit immunoglobulins labeled with fluorescein or colloidal gold particles.

Flavonoids were found mainly in the wall of epidermal cells and to a lesser extent mesophyll cells. Alkaloids, on the other hand, were accumulated in the vacuole of mesophyll cells and idioblasts. These results demonstrate the specificity and sensitivity of immunological methods to

localize plant secondary metabolites. Furthermore, these results indicate the importance of extraplasmic compartments (cell wall and vacuole) in sequestering secondary plant metabolites.

A mes parents devoués: Laurette & (feu) Antonio Brisson

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1. INTRODUCTION

Plants interact with their environment by producing an array of chemicals which are collectively known as secondary metabolites. In contrast with primary metabolites, the latter term was conceived and defined to designate those natural products that seem to play no conspicuous role in the biochemical activities that support plant growth and development. Depending on the metabolic pathways involved in their formation this group may be subdivided as alkaloids, flavonoids, and terpenoids, for example.

The great variety of secondary metabolites and their wide distribution raise the question as to their significance in nature. When one considers the chemical diversity of secondary metabolites, however it seems unlikely that they all possess a similar function. On the other hand, this diversity may represent an "armoury" evolved by plants in response to various environmental pressures (Beart et al. 1985). Several reports have documented the ecological significance of plant secondary metabolites as attractants of pollinators or symbionts; as antimicrobial substances (phytoalexins); as attractants or repellents of insects or herbivores (for reviews, see Bell 1981; Vickery and Vickery 1981); and most recently as chemical signals in the association of Rhizobium spp. (Firmin et al. 1986; Peters et al. 1986; Sadowsky et al. 1988) and Agrobacterium tumefaciens (Bolton et al. 1986; Spencer and Towers 1988; Stachel et al. 1985) with their host plants.

Plant secondary metabolites are known to be toxic not only to other living organisms, but also towards the host plants that produce them (Boller and Wiemken 1986). In addition, it has been reported that several "non-toxic" compounds may inhibit important physiological processes. Plant phenolics, for example, may interfere with seed germination (Mandakumar et al. 1985), photosynthesis (Moreland and Novitzky 1988; Muzafarov et al. 1986; Takahama 1984), mitochondrial phosphorylation (Koeppe and Miller 1974), photophosphorylation (Arntzen et al. 1974; Ravanel 1986), respiration (Ravanel et al. 1981, 1982), auxin biosynthesis and/or its transport (Jacobs and Rubery 1988). In order to maintain the plasmic concentration of a secondary metabolite at a non-toxic level, the expression of secondary metabolism must be coordinated with other cellular functions. This may imply a spatial and temporal organization which regulates the rates of biosynthesis, transport, accumulation and/or degradation. This may be achieved by a spatial arrangement of enzymes, channeling of intermediates and/or compartmentation of the enzymes and their products. The mechanisms for storage, transport and excretion of secondary metabolites have recently been summarized and discussed by Roos and Luckner (1986). In fact, many plants tend to concentrate their secondary metabolites in specialized glandular tissues or storage cells, such as laticifers and idioblasts (for a review, see Wink 1987). In the absence of these structures, toxic secondary metabolites may be separated from the centers

of metabolic activity by their accumulation in nonplasmic compartments. In general, it is believed that lipophilic compounds are located in the extraplasmic space or in vesicles, whereas the hydrophilic ones are usually dissolved in the acidic vacuole (Guern et al. 1987; Luckner 1980; Luckner et al. 1980; Wagner 1982; Wink 1987). Although several reports tend to support this hypothesis, such generalizations are, at present, inconclusive. Despite the significant progress that has been achieved in the biochemistry and enzymology of secondary metabolism, only few attempts have dealt with their intracellular compartmentation. In addition, methods commonly used to study the subcellular localization of a given metabolite usually involve histo-cytochemistry, tracer experiments or cell fractionation. These techniques present a number of limitations and may introduce artefacts that may render the interpretation of results ambiguous. Therefore, the site of accumulation of a specific metabolite detected with the aid of the above techniques should be reconsidered using more rigorous techniques such as in situ spectropnotometry, laser desorption spectrometry or by immunological methods (Manen and Pusztai 1982; Moesta et al. 1982). An ideal technique should allow the detection of the metabolite in a given area of a specific organ, tissue and/or cell. This prompted me to apply immunochemical techniques, which are both sensitive and specific, for the localization of secondary metabolites.

Chrysosplenium americanum Schwein. ex Hooker

(Saxifragaceae) accumulates a number of tri- to penta-O-methylated flavonols (Figure 1.1). These compounds occur as 2'/5'-monoglucosides together with trace amounts of their free aglycones (DeLuca 1984). Their methyl substitution at positions 3, 6, 7, 2', 4', and/or 5' has stimulated the interest in purifying and characterizing the enzymes involved in their O-methylation (DeLuca and Ibrahim 1982, 1985a, 1985b; Khouri et al. 1986) and O-glucosylation (Bajaj et al. 1983; Ibrahim et al. 1987, 1989; Khouri and Ibrahim 1984; Latchinian et al. 1987). While methyl substitution of flavonols increases their lipophilic nature, the presence of a glucosyl residue renders these compounds partially hydrophilic. With our current interest in the localization of secondary metabolites, this dual solubility property prompted me to use C. americanum as an experimental model system.

The madagascan periwinkle, Catharanthus roseus (L.) G. Don (Apocynaceae), contains at least 200 known alkaloids (Mersey and Cutler 1985). This plant is the sole source of two bis-indole monoterpenoid alkaloids, vinblastine and vincristine, which are extensively used in cancer chemotherapy (Cordell 1978a). These dimers are formed by the enzymatic condensation of vindoline and catharanthine (see Misawa et al. 1988 and refs therein), and are found in extremely low concentration in intact tissues, ca. 0.003% of dry weight (Cordell 1978b). Efforts to produce the bis-alkaloids in cultured tissues have met with little success, possibly due to the fact that undifferentiated cells lose their ability to

elaborate and/or accumulate vindoline (DeLuca and Kurz 1988). The absence of vindoline in cultured cells has, therefore, stimulated interest in studying the regulation of its biosynthesis (see DeLuca and Kurz 1988; Misawa et al. 1988 and refs therein). On the other hand, the lack of subcellular localization studies of alkaloids prompted us to determine the site(s) of accumulation of vindoline in C. roseus in order to precisely identify whether its presence may be correlated with specialized cells or tissues.

To date, immunological localization of a hapten presents a number of technical problems, especially the leaching of the antigen during the chemical fixation and dehydration of the material. Nevertheless, molecules such as plant hormones (Skene et al. 1987 and refs. therein), herbicides (Huber and Sautter 1986) and polysaccharides (Lynch et al. 1989; Moore et al. 1986; Moore and Staehelin 1987) have been successfully localized by immunological techniques following their fixation by rapid freezing or by an esterifying agent. As far as we know, these techniques have not been used to localize flavonoids or alkaloids in plants. In the present study, their potential will be evaluated for the localization of plant secondary metabolites at both the tissue and cellular levels using immunofluorescence and immunogold labeling techniques.

Figure 1.1. Major flavonoids of Chrysosplenium americanum.

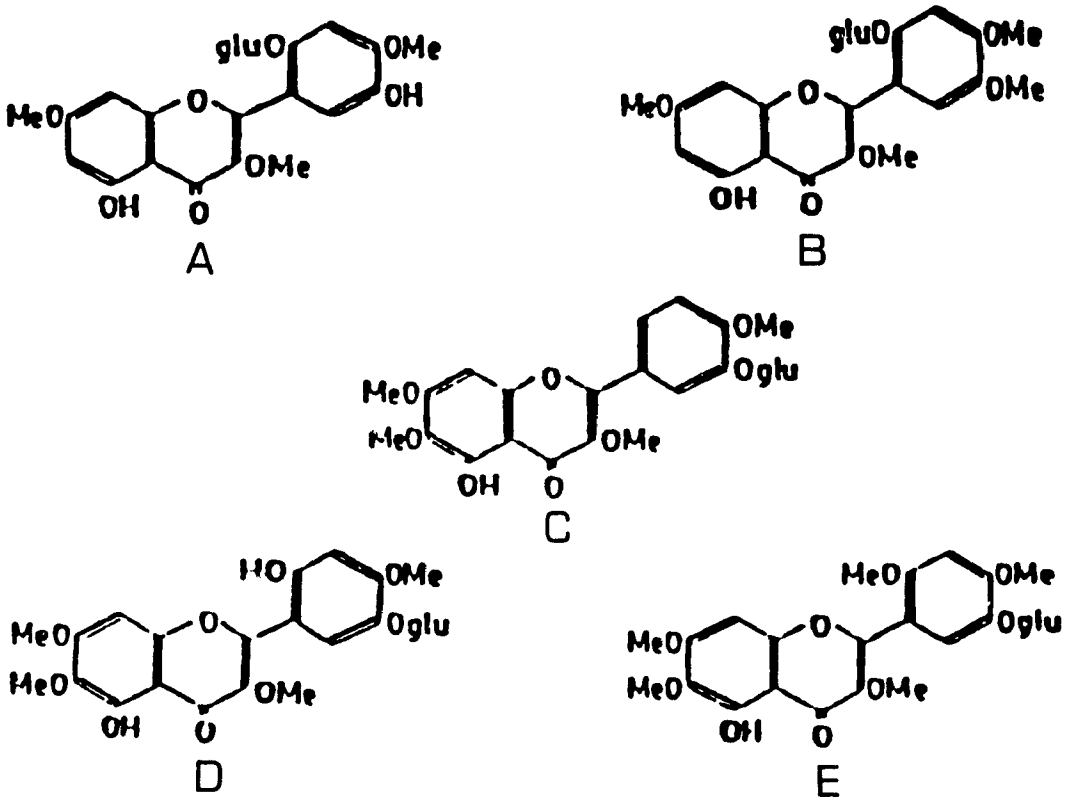
A) 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone-2'-O-glucoside

B) 5,2'-dihydroxy-3,7,4',5'-tetrametoxyflavone-2'-O-glucoside

C) 5,5'-dihydroxy-3,6,7,4'-tetramethoxyflavone-5'-O-glucoside

D) 5,2',5'trihydroxy3,6,7,4'tetramethoxyflavone-5'-O-glucoside

E) 5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone-5'-O glucoside.


gluO
OMe
OH
MeO
O
OMe
OH
O
A
gluO
OMe
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MeO
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OH
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MeO
MeO
O
OMe
OH
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E

II. REVIEW OF LITERATURE

In the last decade, our knowledge of the biochemistry and enzymology of plant secondary metabolites has expanded quite rapidly. This contrasts with the meager number of studies dealing whith localization and intracellular compartmentation of these compounds which are required for the understanding of their physiological and ecological roles. The technical difficulties encountered in localization studies have obviously discouraged detailed investigations. It was considered important, therefore, to focus the following review on the methodological aspects concerning the study of compartmentation of secondary metabolites with special reference to phenolic compounds and alkaloids. The former term is being used in a broad sense, and includes phenylpropanoids, flavonoids and tannins. However, reference to individual classes will be made where appropriate.

The reliability of the methods used for localization studies is obviously based on the precise identification of both the secondary metabolite and the compartment (tissue, cell, organelle) under investigation. In the case of plant secondary metabolites, success has rarely been achieved and in some cases, the interpretation of data may have been seriously flawed. Proper attribution of results requires a general consideration of physiological, ecological, biochemical and technical aspects.

2.1. Physiological and environmental considerations

All plant cells possess the genetic potential to produce and accumulate natural products (Gautheret 1985). However, it is well known that the expression of secondary metabolism may occur exclusively in specific tissues or cells at some well defined developmental stage; i.e. space and time (for reviews see Bohm 1980, 1982; Tuckner 1984; Wiermann 1981). This implies a strong correlation between the various components of secondary metabolism (biosynthesis, transport, accumulation and/or degradation) and cell differentiation or tissue specialization. Biosynthesis, for example, is frequently triggered by environmental stress and/or elicitors, as well as other conditions known to accelerate the development of the plant (for reviews see Darvill and Albersheim 1984; DiCosmo and Misawa 1985; Dixon 1986; Eilert 1987). Moreover, the distribution of secondary metabolites within a plant organ may change significantly depending on its age, its physiological state, and the prevailing environmental factors (McClure 1975).

With the extensive use of <u>in vitro</u> culture, an enormous body of information is now available showing the effect of environmental conditions (light, temperature, nutrition, etc.) on the expression and repression of plant secondary metabolism. For example, the effect of light on the biosynthesis of flavonoids (for reviews see Ebel and Hahlbrock 1977; Hahlbrock and Scheele 1989; Ibrahim 1987; Hinderer and

Seitz 1988) and alkaloids (for review see DeLuca and Kurz 1988) has been well studied. Although illumination generally causes an increase in the accumulation of secondary metabolites, its inhibitory effect has been reported for the production of polyphenols and anthraquinones (see Ibrahim 1987 and refs therein). Many reports have also documented the importance of the quality and quantity of light (Beggs et al. 1985; Duell-Pfaff and Wellmann 1982; Fritzemeir et al. 1983; Kreuzaler and Hahlbrock 1973; Moehle and Wellmann 1982; Moehle et al. 1985; Schmelzer et al. 1988; Schroder et al. 1979; Steinitz and Bergfeld 1977; Wellmann 1971). The stimulating effect of UV irradiation has been especially well studied in parsley cell cultures, the model system used by the Freiburg group to study the regulation of flavonoid biosynthesis (see Chappell and Hahlbrock 1984; Hahlbrock 1977; Hahlbrock and Scheele 1989; Hahlbrock et al. 1976; Jones 1984; Kuhn et al. 1984 and refs therein). Furthermore, it has been shown that UV-induced secondary metabolite synthesis could be prevented or enhanced by illumination with light of different quality. Therefore, the influence of light, as well as other environmental factors depends on both the plant material and the metabolite under investigation. A complete coverage of these factors is beyond the scope of this review. However, the use of light was given as an example to simply illustrate the complexity of the system and the necessity to consider, in any localization study, the developmental and metabolic parameters as well as the environmental conditions.

2.2. Biochemical considerations

The diversity of plant secondary metabolites usually involves differences in their chemical structure, substitution pattern, molecular weight and solubility. Compounds with a planar configuration and small molecular weight (<500), such as phenylpropanoids and polar flavonoids, are freely diffusible in biological systems. Consequently, such metabolites may be leached out during tissue processing or redistributed among the different compartments and hence, preclude accurate localization (see Burmeister and Hösel 1981; Roos and Luckner 1986 and refs therein). It has been shown that isolated broken chloroplasts are more liable to bind flavonoid compounds (Charrière-Ladreix and Tissut 1981) or the enzymes involved in their methylation (Thresh and Ibrahim 1985) than the intact organelle. Such results, as well as other earlier reports on the subcellular localization of flavonoids (for review, see Ibrahim et al. 1986) call for a re-evaluation of these findings. Despite this experimental problem, the diffusion of metabolites was circumvented in some histo/cytological studies by the use of special chemical or physical fixation methods or by a rapid dehydration procedure. For example, phenolic compounds can be stabilized in the tissue by caffeine or ferric chloride (Bal and Savory 1980; Brisson et al. 1977; Mueller and Greenwood 1978; Zobel 1986; Zobel and Brown 1988). The esterification of plant growth regulators with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

was achieved to prevent their diffusion (see Skene et al. 1987 and refs. therein) and therefore, allowed their localization by immunocytochemistry. Alternatively, physical fixation involving cryofixation and cryosubstitution was used to reduce diffusional artifacts (Fisher and Housley 1972; Herman 1988; Saunders et al. 1977; Zavala and Brandon 1983). In the study of Saunders et al. (1977), the detection of dhurrin in vacuoles of *Sorghum bicolor* by autoradiography was achieved by minimizing the period of dehydration with the use of 2,2-dimethoxy-propane.

Another limitation to the unequivocal localization of secondary metabolites is their common occurrence in very low concentrations. Furthermore, many metabolites share common chemical structures and physical properties (fluorescence, colour reaction) that restrict their characterization in localization studies. Therefore, the techniques to be used in the localization of these compounds should be sensitive and specific.

2.3. Technical considerations

Table 2.1 lists a selection of published papers to illustrate the common methodology used by plant investigators to localize plant phenolics. As indicated in this table, our knowledge of tissue, cellular and subcellular localization of secondary metabolites has mostly been derived from microscopic observations and/or biochemical analysis of fractionated plant material. Depending on the metabolite to

Table 2.1. Experimental methodology commonly used to localize plant phenolics.

METABOLITE	PLANT SPECIES	TISSUE	SITE OF ACCUMULATION	Methodology: MICROSCOPY	Methodology: CHROMATOGRAPHY	REFERENCES
PHENOLICS (unidentified)	*Allium cepa*	root	EL & HL/ W	BF, FL, EM	---	Peterson *et al.* 1978
	Elchhornia crassipes	leaf	SEL & ML/ V	BF	---	Martyn *et al.* 1983
	Musa acuminata	root	VT/ I / V	EM	---	Mueller & Greenwood 1978
	Gossypium hirsutum	root	VT/ I / V	EM	---	
	Prunus avium	shoot	VaT/ W, P & M	BF, EM	---	Schmid *et al.* 1984
	Rubus chamaemorus	root	VT/I/W, C & M	BF, EM	---	Bal & Savory 1980
	Solanum tuberosum	tuber	W	BF	HPLC	Ampomah & Friend 1988
PHENOLIC ACIDS	*Avena sativa*	leaf protoplast	W	FL	---	Knogge & Weissenbock 1976
	Hordeum vulgare	seed	A/ W	FL;S	HPLC	Fulcher *et al.* 1988
	Triticum aestivum	seed	A/ W	FL;S	TLC	
	Triticum aestivum	seed	A/ W	BF, FL;S	HPLC	Fulcher *et al.* 1972
	Triticum aestivum	coleoptile	VT/ VC/ W	BF	TLC	Whitmore 1974
	Melilotus alba	leaf	V	---	TLC	Oba *et al.* 1981
	various gramineae	protoplast V organs	VT/ VC/ W	FL	---	Harris & Hartley 1976
NAPHTOQUINONES	*Lithospermum erythrorhizon*	cultured cells	extraplasmic, ER	BF, EM	TLC	Tabata *et al.* 1982
ANTHOCYANINES	*Bryophyllum crenatum*	leaf	E	---	TLC	Tronchet 1968
	Raphanus sativus	leaf	E	---	HPLC	Strack *et al.* 1985
	Secale cereale	leaf protoplast	M	---	HPLC	Strack *et al.* 1982
	Vitis spp.	fruit vacuole	SE/ V	BF	HPLC	Moskowitz & Hrazdina 1981
	Various plant species	vacuole	E/ V	BF	---	Wagner & Siegelman 1975

TISSUE
A : Aleurone layer
EL : Epidermal layer
HL : Hypodermis
ML : Mesophyll layer
SEL: Subepidermal layer
VaT: Vascular tissue
VT : Various tissue

CELL
I : Idioblast
VC: Various cell

Methodology
BF: Bright field microscope
Fl: Fluorescence microscope
EM: Electron microscope
S : Spectrophotometry

ORGANELLE
C Chloroplast
ER Endoplasmic reticulum
M Membrane
P Plasmalemma
V Vacuole
W Wall

Table 2.1. Experimental methodology commonly used to localize plant phenolics. (continued).

METABOLITE	PLANT SPECIES	TISSUE	SITE OF ACCUMULATION	Methodology: MICROSCOPY	Methodology: CHROMATOGRAPHY	REFERENCES
FLAVONOIDS						
Diverse aglycones	various plants	bud, leaf	exudate	see text		
Diverse glycosides	various plants	C (leaf)	C	---	TLC	Saunders & McClure 1976
flavonols	various plants	leaf	E/ V	FL	Sp	Tissut & Ravanel 1980
flavones	*Silene pratense*	leaf VT/ C	E/ C & V	FL	HPLC	Niemann *et al.* 1983
malonylated flavonols	*Petroselinum hortense*	cultured cells	V	FL	TLC	Matern *et al.* 1983
isovitexin	*Silene pratensis*	leaf & petals	E/ V	FL;S	---	Brederode & Kooten 1983
kaempferol and/or quercetin and their derivatives	*Raphanus sativus*	leaf	E	FL;S	HPLC	Strack *et al.* 1985
	Pisum sativum	leaf protoplast	E/ V	FL	TLC	Weissenbock *et al.* 1986
	Vicia faba	leaf	E/ V	FL	TLC	Viestra *et al.* 1982
	Vicia faba	leaf	E/ V	FL;S	---	Schnabl *et al.* 1986
	Allium cepa	leaf	E/ V	FL;S	HPLC	Weissenbock *et al.* 1982
	Vitis spp.	fruit	V	---	HPLC	Moskowitz & Hrazdina 1981
	Glycine max	leaf	E	---	HPLC	Coslo & McClure 1984
apigenin and its derivatives	*Avena sativa*	C	C	---	TLC	Weissenbock & Schneider 1974
	Avena sativa	leaf protoplast	E & M	---	HPLC	Knogge & Weissenbock 1986
	Secale cereale	leaf protoplast	E & M	---	HPLC	Strack *et al.* 1982
naringenin and its derivatives	*Lycopersicum esculentum*	fruit	cuticle	---	TLC	Hunt & Baker 1980

be detected, the sensitivity of these methods varies from low to moderate. Such methods may be used to indicate the major sites of accumulation (Guern et al. 1987) and the results obtained should be interpreted with caution.

2.3.1. Conventional histochemical methods

Earlier studies on tissue and cellular distribution of secondary metabolites have involved light and electron microscopic observations. One of the objectives of these studies was to establish the relationship between morphological and biochemical heterogeneity. However, despite the fact that histochemical methods may be used to identify the major classes of compounds, the interpretation of results has always been limited by the method of preparation of the plant material, as well as the techniques used in metabolite identification. Nevertheless, the use of microscopy continues to be an important tool for the localization of coloured (naphthoquinones, anthocyanins), fluorescent (certain phenylpropanoids, flavonoids and alkaloids) and electron dense compounds (tannins) at the tissue and cell levels. Since the first detection of anthocyanins in the vacuole by Poltis in 1903, histochemical investigations continue to be of interest to plant physiologists, either in the study of tissue specificity or to elucidate the regulation of secondary metabolite synthesis. Recently, Nozzolillo and Ishikura (1988) have examined the distribution of anthocyanoplasts, the vacuolar structures in which anthocyanins are concentrated in

different plant species. Their observations seem to suggest that these structures derive from the endoplasmic reticulum by the coalescence of transport vesicles. Similar conclusions were previously postulated for these compounds (Parham and Kaustinen 1977; Pecket and Small 1980; Sinnerby- Forsse et al. 1987; Small and Pecket 1982) as well as for tannins (Baur and Walkinshaw 1974; Felker et al. 1984; Ginzburg 1967). Ultrastructural observations seem to indicate that vesicles, originating from ER, are also involved in the transport and excretion of naphthoquinones in the cells of Lythospermum erythrorhyzon (Tabata et al. 1982) as well as in the excretion of flavonoid aglycones in glandular hairs of various plant species (Charriere-Ladreix 1973, 1975, 1976; Fähn 1979; Wollenweber 1984; Wollenweber and Dietz 1981). Taken together, these several lines of observations seem to substantiate the importance of vesicles for the intracellular transport of secondary metabolites. However, definite proof awaits the development of more accurate methods.

On the other hand, fluorescent compounds may be further characterized by spectroscopic methods and/or phytochemical analysis. Both techniques have successfully been used in the identification of phenolic acids associated with the aleurone cell walls of wheat grains (Fulcher et al. 1972; 1988) and of flavonoid derivatives found in fluorescing vacuoles of Allium cepa (Peterson et al. 1978; Weissenbock et al. 1987), Petroselinum hortense (Matern et al. 1983; 1986), Silene pratense (Niemann et al. 1983) and Vicia faba (Schnabl et al.

1986).

Colourless metabolites may be identified *in situ* by the use of histochemical reagents. However, their potential toxicity to plant cells, as well as their specificity for the metabolite under investigation should be evaluated. It is not surprising, therefore, that despite the specificity of the alkaloid reagents, ceric ammonium sulfate and bismuth subnitrate, their application was restricted because of their damaging effect on cellular integrity (Yoder and Mahlberg 1976). In addition, several non-specific histochemicals (e.g. phloroglucinol, nitroso reagent, potassium iodide, caffeine, etc.) have commonly been used for the localization of secondary metabolites. For example, the nitroso reagent, a common phenolic indicator, has been successfully applied in the localization of proanthocyanidins, the major constituent of gymnosperm tissues (Stafford 1988; Stafford *et al*. 1987, 1989). Therefore, histochemical studies should be complemented with phytochemical analysis in order to provide more precise information.

2.3.2. Biochemical methods

The recent progress in biochemical analysis (differential centrifugation, TLC, GC, HPLC, etc.) offers a greater potential for the localization of secondary metabolites, and their distribution in different tissues, cells, and/or organelles. A combination of these methods can be used to establish the chemical identity of a compound as well as the

site(s) of its accumulation. HPLC analysis has recently been used to recognize two groups of plants differing by their tissue-specific flavonoid metabolism. The first group was characterized by a high flavonoid concentration in leaf epidermis as found in Avena sativa (Knogge and Weissenbock 1986; Weissenbock 1988), Silene pratensis (Niemann et al. 1983) and Sinapis alba (Wellmann 1974). In the second group, the constituent flavonoids were found to occur in both epidermal and mesophyll tissues in varying proportions (Tissut and Ravanel 1980). Similarly, the chromatographic analysis of plant tissue effusates (i.e. tissues dipped in organic solvents for one or two minutes) has indicated that the solubilized material contains high concentrations of lipophilic compounds, such as terpenoids and flavonoid aglycones (for reviews, see Wollenweber 1984; Wollenweber and Dietz 1981). Whereas this method can be applied to the identification of "surface" metabolites, it does not reveal the exact site(s) of their accumulation.

Recent advances in fractionation techniques have allowed the separation of different plant cells and organelles with the aim to localize secondary metabolites at the cellular and subcellular levels. Consequently, the chemical analysis of leaf tissues has suggested the importance of idioblasts and laticifers in the storage of alkaloids and phenolic compounds (for review, see Wink 1987). On the other hand, the recent progress in the isolation of intact cellular compartments has demonstrated the accumulation of various secondary

metabolites, including cyanogenic glucosides, alkaloids and phenolic compounds in the vacuole (for reviews see Boller and Wiemken 1986; Luckner 1979, 1984; Marty et al. 1980; Matile 1978; 1884). However, the reliability of these biochemical methods depends primarily on the purity and precise identification of the tissue, cell and organelle under investigation.

2.3.3. Immunological techniques

As early as 1941, Coons and his collaborators have shown that antibodies could be conjugated to some markers without losing their affinity or specificity for their corresponding antigens (Pearse 1980). This finding has opened up a new field of histo-cytochemistry and allowed cell biologists to reveal specific antigenic sites over well preserved, identifiable structures. To date, the reliability of immunological localization is well established through numerous applications in basic and applied research. However, the application of immunolocalization techniques has been hampered due to difficulties inherent to plant tissue, especially the presence of cell wall and vacuoles (Burmeister and Hösel 1981). Nonetheless, recent examples have demonstrated the wide range of their applications to plant tissues (see Tables 2.2 and 2.3).

2.3.3.1. General principle

Immunological localization of an antigen is simply based on the precipitation of an antibody from a relatively

Table 2.2. Selected examples of metabolites localized by immunofluorescence techniques.

FUNCTIONAL PROTEINS	PLANT SPECIES	SITE OF ACCUMULATION	REFERENCES
Amylase	*Hordeum vulgare*	aleurone	Jacobsen & Knox 1973
	Hordeum vulgare	aleurone	Jones & Chen 1976
	Hordeum vulgare	starchy endosperm & scutellum	Lauriere *et al.* 1986
Auxin transporter	Pisum sativum	plasma membrane	Jacobs & Gilbert 1983
Calmodulin	*Allium cepa* & *Pisum sativum*	mitotic spindle	Wick *et al.* 1985
		mitotic spindle	Wick & Duniec 1986
Cellulolytic enzymes	Phaseolus vulgaris	abscission zone	Durbin *et al.* 1981
	Phaseolus vulgaris	abscission zone	Sexton *et al.* 1981
Chalcone synthase	Petroselinum crispum	epidermis	Schmelzer *et al.* 1988
	Spinacia oleracea	epidermis	Beerhues *et al.* 1988
	Tulipa sp.	(anther) tapetum	Kehrel & Wiermann 1985
Coniferyl glucosyl-transferase	Picea abies	cytosol	Schmid *et al* 1982
ß-glucosidase	Cicer arietinum	wall	Burmeister & Hosel 1981
	Picea abies	wall	Marcinoswski *et al* 1979
	Trifolium repens	wall	Kakes 1985
Glutamine synthase	Lathraea sp.	chloroplast	Hirel *et al.* 1982
	Spinacia oleracea	cytosol	Thalouarn *et al.* 1987
Lipid acylhydrolase	Solanum tuberosum	epidermis	Vancanney *et al.* 1989
Lipoxygenase	*Glycine max*	variable	Vernosy *et al* 1983
Malate dehydrogenase	Citrullus vulgaris	epidermis	Sautter & Hock 1982
Nitrate *reductase*	*Glycine max*	cytosol	Vaughn *et al* 1984
Peroxydase	Solanum tuberosum	wall	Espelie *et al.* 1986
Phenylalanine ammonia lyase	*Tulipa* sp.	tapetum	Kekhrel & Wiermann 1985
PEP carboxylase	Various C3/C4	chloroplast	Perrot *et al.* 1981, 1982, 1984 Madhavan & Smith 1984
Phosphorylase	Solanum tuberosum	amyloplast/cytosol	Schneider *et al.* 1981
	Spinacia oleracea	mesophyll	Steup & Schachtele 198
Photosystem	Vicia faba	epidermis	Zemel *et al.* 1988
Pyruvate dikinase	Triticum aestivum	aleurone & pericarp	Aoyogi & Chua 1988
Rubisco	Various plant species	chloroplast	Bauwe 1984
		chloroplast	Hattersley *et al.* 1977
		chloroplast	Madhavan & Smith 1982
		chloroplast	Reed & Chollet 1985
		chloroplast	Zemel & Gepstein 1985
Urease	Canavella sp.	cytosol/ intercellular space	Murray & Knox 1971
Uricase	Glycine max	all cells	Bergmann *et al.* 1983
STORAGE PROTEINS LECTINS			
Vicilin & legumin	*Vicia faba*	protein body	Graham & Gunning 1970
Agglutinin	*Triticum aestivum*	epidermis	Miskind *et al.* 1982
Lectin	various plant species	various cells	Jeffre & Yeoman 1981
HAPTENS			
ABA	*Vicia faba*	epidermis	Curvetto *et al.* 1986
Atrazine	*Zea mays*	vascular tissue	Huber & Sautter 1986
CK	*Zea mays*	cytosol	Zavala & Brandon 1983

Table 2.3. Selected examples of metabolites localized by immunocytochemistry.

FUNCTIONAL PROTEINS	PLANT SPECIES	SITE OF ACCUMULATION	REFERENCES
ATPase	*Zea mays*	vacuole	Harley & Taiz 1988
Chalcone synthase	*Spinacia oleracea*	cytosol	Beerhues *et al.* 1988
	Fagopyrum esculentum	endoplasmic reticulum	Hrazdina *et al.* 1987
Chitinase & glucanase	*Phaseolus vulgaris*	vacuole	Mauch & Staehelin 1989
Cytochrome P450	*Persea americana*	chloroplast (grana) & endoplasmic reticulum	Bourett *et al.* 1989
Cytochrome b559	*Spinacia oleracea*	chloroplast (grana)	Rao *et al.* 1988
Extensin	*Daucus carota*	cell wall	Stafstrom & Staehelin
	Glycine max	cell wall	Cassab & Varner 1987
	Pisum sativum	cell wall	Hermoso *et al.* 1989
Fructose 1-6 biphosphatase	*Pisum sativum*	stroma & thylakoid	Hermoso *et al.* 1989
Glutamate denydrogenase	*Chrorella sorokiniana*	plastid	Prunkard *et al* 1986
Glutamine synthethase	*Lycopersicon esculentum*	chloroplast	Botella *et al* 1988a
GOGAT system	*Lycopersicon esculentum*	chloroplast	Botella *et al.* 1988b
Isocitrate lyase	*Gossypium hirsutum*	glyoxysome	Doman & Trelease 1985
Leg-haemoglobin	*Pisum sativum*	cytosol	Robertson *et al.* 1984
Nitrate reductase	*Chlamydomonas sp*	pyrenoid	Lopez-Ruiz *et al.* 1985
	Spinacia oleracea	chloroplast	Kamachi *et al* 1987
	Zea mays	cytosol	Vaughn & Campbell 1988
Nitrogenase	*Elaeagnus pungens*	nodule vesicle	Sasakawa *et al.* 1988
Pigment binding prot.	*Zea mays*	stroma	Verbele *et al.* 1989
Pathogenesis proteins	*Lycopersicon esculentum*	intercellular space	Vera *et al.* 1988
	Nicotiana tabacum	intercellular space	Hosokawa & Ohashi 1988
Phosphoribulose kinase	*Cyanophora*	cyanelle	Mangeney *et al.* 1987
	Glaucocystis		
Phytochrome			
	Avena sativa	cytosol & membrane	McCurdy & Pratt 1986
	Avena sativa	cytosol & membrane	Verbelen *et al.* 1982
Propyl hydroxylase			
	Chlamydomonas reinhardi	endomembranous & wall	Andrea *et al* 1988
Rubisco			
	Capsicum sp.	chloroplast	Chenicliet *et al.* 1988
	Chlamydomonas	chloroplast	Lacoste Royal & Gibbs
	Chlorella pyrenoidosa	chloroplast	McKay & Gibbs 1989
	Citrofortunella mitis	chloroplast	Suire *et al.* 1988
	Hordeum vulgare	chloroplast	Mangeney & Gibbs 1987
	Nicotiana tabacum	chloroplast	Shaw & Henwood 1985
	Pelargonium hortorum	chloroplast	Vaughn 1987
Starch phosphorylase	*Nicotiana tabacum*	amyloplast/cytosol	Brisson *et al* 1989
Uricase	*Glycine max*	peroxisome	Bosch & Newcomb 1986
	Glycine max	peroxisome	Nguyen *et al.* 1985

Table 2.3. Selected examples of metabolites localized by immunocytochemistry (continued).

STORAGE PROTEINS	PLANT SPECIES	SITE OF ACCUMULATION	REFERENCES
phaseolin	*Phaseolus vulgaris*	endoplasmic reticulum, protein body, Golgi	Greenwood & Chrispeels 1985
vicilin & legumin	*Pisum sativum*	protein body	Craig & Miller 1981
	Vicia faba	protein body	Nieden *et al.* 1982
unidentified	*Phaseolus vulgaris*	endoplasmic reticulum	Baumgartner *et al.* 1980
	Pisum sativum	membrane	Craig & Miller 1981
	Pisum sativum	cytosol	Harris & Craig 1985
LECTINS			
agglutinin	*Triticum aestivum*	protein body	Miskind *et al.* 1982
concanavalin A	*Bauhinia purpurea*	golgi	Herman & Shannon 1984, 1986
phytohemagglutinin	*Phaseolus vulgaris*	protein body	Greenwood *et al.* 1984
	Phaseolus vulgaris	endoplasmic reticulum golgi	Greenwood & Chrispeels 1985
HAPTENS			
Growth regulators			
ABA	*Chenopodium*,	plastid	
	Daucus carota	wall	Sossountzov *et al.* 1986
	Lycopersicon	wall & cytosol	Skene *et al.* 1987
			Bertrand *et al.* 1989
CK	*Lycopersicon*	wall	
	Zea mays	cytosol	Eberle *et al.* 1987
			Zavala & Brandon 1983
Polysaccharides			
rhamnogalacturonan	*Acer pseudoplatanus*	wall	Moore *et al.* 1986
xyloglucans	*Trifolium pratense*	wall	
	Trifolium pratense	wall	Moore & Staehelin 1988
	Acer pseudoplatanus	wall	Lynch *et al.* 1989
	Acer pseudoplatanus	wall	Moore *et al.* 1986
			Staehelin *et al.* 1988
unidentified	*Zea mays*	wall	Hahn *et al.* 1989

concentrated solution over a tissue section containing the antigen in reactive form. The detection of the immune complex is then visualized by a direct or an indirect method. The former involves a reaction between the labeled antibody and its corresponding antigen. Alternatively, in the indirect method, the section is first incubated with an unlabeled antibody followed by a secondary reaction implying the interaction of immunoglobulins with labeled compounds (Figure 2.1). Protein A or anti-antibodies are commonly used as the secondary reagent after being tagged with specific markers. This step introduces an amplification factor which enhances the sensitivity of the technique. In fluorescence microscopy, the most commonly used markers are fluorescein and rhodamine. An indirect immunoperoxidase staining was recently used to localize abscisic acid in the buds of Chenopodium polyspermum (Sotta et al. 1985), and chalcone synthase, the key enzyme of flavonoid biosynthesis, in epidermal cells of parsley leaves (Schmelzer et al. 1988). Chalcone synthase was also detected in light microscope by the red color of the gold marker in anthocyanin-producing cells of buckwheat hypocotyls (Hrazdina et al. 1987a, 1987b). A pre-embedding labeling procedure allows the visualization of gold particles in light microscopy since the marker is not restricted to the cut surface (Raikhel et al. 1984).

For electron microscopy, ferritin and peroxidase were occasionally used as markers for the localization of phosphoenolpyruvate carboxylase (Gadal et al. 1983, Perrot et

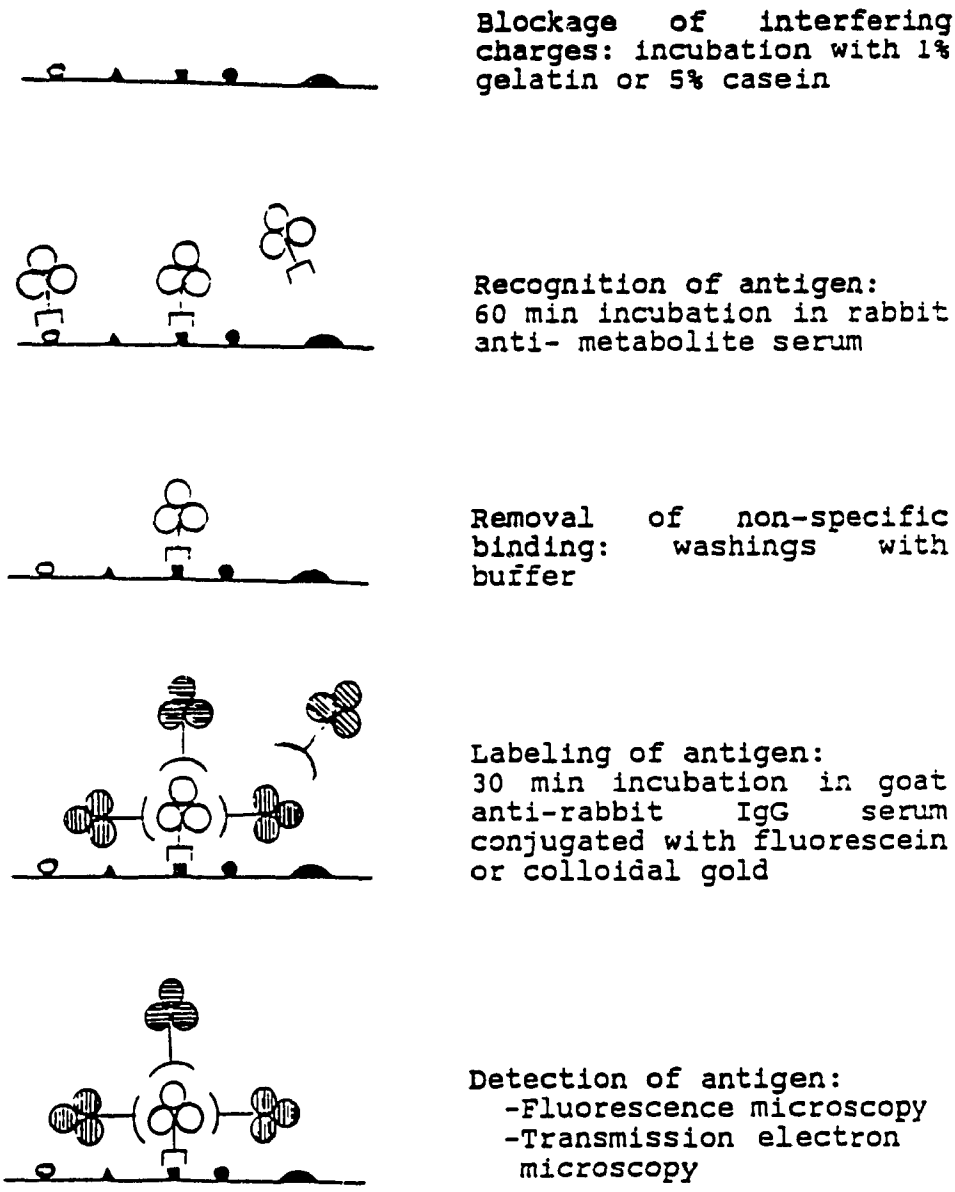


Figure 2.1. Indirect immunolabeling technique

al. 1981). Colloidal gold particles have been extensively adopted as the best marker in electron microscopy (see examples in Table 2.3) since they show little tendency for non-specific binding (Herman 1988). Futhermore, immunolocalization with gold particles is generally considered to be more precise and quantitative since this marker is monodisperse (Bendayan and Zollinger 1983, Brewin et al. 1987, Vaughn 1987).

2.3.3.2. Preparation of plant tissue

The preparation of tissue samples represents a compromise between morphological preservation, retention of antigenicity and maintenance of protoplasmic integrity (Herman 1988; Wick and Deniec 1986). Therefore, freshly prepared, immunostained sections are occasionnally used in localization studies (Curvetto et al. 1986, Madhavan and Smith 1982, Zemel et al. 1988). Alternatively, cells or tissues are fixed, dehydrated, embedded, then sectioned before being observed by light and/or electron microscopy. Although the conventional use of glutaraldehyde and OsO_4 helps preserve the cellular ultrastructure, it has been reported that these fixatives may reduce the antigenicity of the compound (Herman 1988). Moreover, the reaction of plant tissues with glutaraldehyde results in a yellow-green fluorescence that might interfere with the fluorescence of fluorescein (Jeffree et al. 1982). A number of fixation procedures are now available and have recently been discussed by Wick and Duniec (1986). Optimal

fixation procedures depend on the nature of the antigen and the plant material under investigation. However, better preservation of antigenicity seems to be achieved when tissues are processed at low temperature (Brewin et al. 1987). The intracellular distribution of labile cellular components is also better preserved by the use of low temperature preparative techniques (Nishizawa and Mori 1989; Zavala and Brandon 1983). Therefore, following cryofixation or chemical fixation, polar acrylic resins (e.g. Lowicryl K4M, LR White) are preferred to epoxy resins in such studies.

2.3.3.3 Immunolabeling

Immunological labeling of an antigen may be accomplished using various procedures before or after tissue embedding. Pre-embedding labeling usually involves cryofixation and cryosectionning. The absence of embedding matrix increases the concentration of antigens accessible for the immune reaction. In addition, the use of this procedure offers excellent antigen retention and consequently, high density label, as demonstrated in many studies (Cresti et al. 1987; Greenwood and Chrispeels 1985; Greenwood et al. 1984; Lancelle et al. 1987; Raikhel et al. 1984). Despite these advantages, this procedure could not be employed in certain studies, such as the localization of chalcone synthase in hypocotyls of buckwheat, because of the lack of membrane preservation (Hrazdina et al. 1987). This problem has also been reported with the localization of agglutinin (Raikhel et al. 1984),

cytochrome f (Shaw and Henwood 1985), cytokinin (Zavala and Brandon 1983), nitrate reductase (Vaughn and Campbell 1988), phaseolin (Baumgartner et al. 1980) and phytochrome (McCurdy and Pratt 1986). Consequently, where possible the post-labeling technique is considered the simplest, reproducible method (Herman 1988). The various protocols of this technique are described in many textbooks (e.g. see Bullock and Petrusz 1982; Pearse 1980; Sternberger 1974).

Another important aspect of immunological studies deals with the level of background and non-specific binding. The specificity of both antibody and staining should be systematically assessed by a series of controls. The non-specific adsorption of immunoglobulins can be detected by an incubation with either preimmune serum or with immune serum that had previously been incubated with its corresponding antigen. The non specific adsorption of fluorescence or gold complexes is generally revealed by the omission of primary serum.

2.3.3.4. Applications to plant tissues

Two decades ago, immunofluorescence techniques were primarily applied to study the distribution of pollen antigens on the outer surface of pollen grains (Knox 1971), and of the storage proteins, vicilin and legumin, in bean cotyledons (Graham and Gunning 1970). Ten years later, the high specificity of the immune reaction was combined with the resolution of transmission electron microscopy to study the

distribution of storage proteins and lectins in the leguminosae (Baumgartner et al. 1980; Craig and Millerd 1981, 1982, Manen and Pusztai 1982; Nieden and Neumann 1982). Considering the rather coarse resolution of light microscopy and the problems arising from autofluorescence of plant constituents, Jeffree et al. (1982) have surmised that immunoelectron microscopy will eventually replace immunofluorescence. However, fluorescence observations have the advantage of examining whole cells in three dimensions (Lloyd 1987). In addition, fluorescence microscopy allows fast screening of many cells in various tissues and at different stages of differentiation, thereby adding spatial and temporal dimensions in the interpretation of results. Consequently, to date, both techniques are widely utilized by plant investigators in order to obtain an overall picture of the distribution of the antigen at the tissue and cell levels.

In that respect, recent articles have listed the variety of proteins that were localized by immunofluorescence (Schmid and Grisebach 1986) and immunogold (Herman 1988) labeling. Proteins that are higly conserved among various plant species have received special attention (see Tables 2.2 and 2.3). These include ribulose 1,5-biphosphate carboxylase (Bauwe 1984; Hattersley et al. 1977; Lacoste-Royal and Gibbs 1987; Shaw and Henwood 1985; Vaugh 1987; Zemel and Gepstein 1985), phosphoenolpyruvate carboxylase (Perrot et al. 1981; Perrot-Rechenmann et al. 1982, 1984; Reed and Chollet 1985) and tubulin (for reviews see Fowke et al. 1984; Lloyd 1987). Other

enzymes have been localized as well as storage proteins and lectins.

Compounds having a molecular weight smaller than 1000, known as haptens, have rarely been localized by immunological techniques (see Tables 2.2 and 2.3). These haptens need to be covalently coupled to a protein in order to stimulate antibody production. In fact, plant growth regulators and plant secondary metabolites have been succesfully conjugated to proteins via carboxyl, hydroxyl, phenolic or ketone moities (for a review of the conjugation methods see Erlanger 1980). Antisera against phytohormone-protein conjugates have been obtained and are being used commercially in radioimmunoassays. Most of them have been developed by Weiler and his collaborators (Atzorn and Weiler 1983, Mertens and Weiler 1983; Weiler 1980, 1983, 1984; Weiler and Mansell 1980; Weiler and Zenk 1976; Weiler et al. 1980). Similarly, a range of radioimmunoassays has been produced for a number of secondary metabolites such as digoxin (Weiler and Zenk 1976), limonin (Weiler and Mansell 1980), naringin (Jourdan et al. 1985a, 1985b), senosides (Artzorn et al. 1981), solasodine (Weiler et al. 1980) and vindoline (Westekemper et al. 1980; Lapinjoki et al. 1987).

The immunolocalization of these haptens presents certain difficulties related to their low abundance in plant tissues as well as their solubility in the solvents normally used in tissue preparation (Skene et al. 1987). However, despite these problems, abscisic acid (ABA) and cytokinin (CK) were

successfully immunolocalized in plant tissues following physical or chemical fixation (Bertrand et al. 1989; Skene et al. 1987; Sossountzov et al. 1986; Sotta et al. 1985; Zavala and Brandon 1983). Zavala and Brandon (1983) have demonstrated the absence of cytokinin in the root quiescent center of Zea mays by immunofluorescence and immuno-gold labeling of cryofixed material. In addition, the parietal accumulation of ABA was demonstrated in various organs and its uneven distribution was shown within these tissues (Bertrand et al. 1989; Skene et al. 1987; Sossountzov et al. 1986; Sotta et al. 1985). These studies allowed plant researchers to correlate some physiological events (such as water stress and senescence) to the distribution of plant growth regulators in the plant.

3. MATERIAL AND METHODS

3.1. Plant Material

Chrysosplenium americanum was collected from Sutton Junction or Huntington (Province of Québec) and maintained under greenhouse conditions simulating its natural habitat. These included their transfer to wet bog soil under diffuse light of low intensity and cool temperature (ca. 15-20 °C).

Seeds of Catharanthus roseus plants were obtained from the Plant Biotechnology Institute, Saskatoon and were grown under greenhouse conditions. Leaves from young seedlings and mature plants were used in this study.

3.2. Protoplasts Preparation

Young leaves of Chrysosplenium and Catharanthus were deveined, immersed in an osmoticum and sliced into strips of about 0.5 mm in width. The osmoticum consisted of 0.5 M mannitol in 25 mM Mes-Hepes buffer (pH 6.5) for C. americanum and 0.7 M sorbitol and 3 mM $CaCl_2$ in 3mM Mes buffer (pH 6.0) for C. roseus. Leaf strips were washed twice in the osmoticum and then immersed in the enzyme solution. Chrysosplenium leaf strips were incubated for four hours in a mixture of 0.4% cellulase Onozuka R-10 and 1.5% macerozyme R-10. Catharanthus protoplasts were obtained after an overnight digestion of leaf segments with a mixture containing 1% cellulase, 0.5% driselase and 0.25% macerozyme. Protoplasts prepared from both plant species were separated from undigested material by filtration through nylon cloth (300 mesh size) and purified

by centrifugation as described by Evans and Cocking (1977).

The use of differential centrifugation and various density gradients of sorbitol, sucrose, Ficoll, Percoll or dextran (Alibert et al. 1982; Mersey and Cutler 1986; Sharma and Strack 1985; Weissenbock and Knogge 1986) was investigated in the present study in order to obtain enriched fractions of epidermal, idioblast or mesophyll protoplasts. Fractions were then evaluated using bright field and fluorescence microscopy.

3.3. Histochemical Localization

3.3.1. Flavonoids

Various phenolic reagents such as aqueous aminoborinate (0.1%), $AlCl_3$ (1% and 15%), $FeCl_3$ (3%), NaOH (0.1N) and NH_4OH were tested with standard phenolic acids and known flavonoids isolated from Chrysosplenium for studying their fluorescence behavior. Histochemicals were directly applied on the section and examined with a Zeiss photomicroscope equipped with epifluorescence. Flavonoids were previously purified by M. Schwec and E. De Carolis using TLC and HPLC (see section 3.5). Table 3.1 shows that after treatment with ferric chloride, flavonoids of Chrysosplenium can be easily distinguished from phenolic acids by their lack of fluorescence. Furthermore, flavonoids can be classified according to their histochemical reactions to three groups, namely, compounds A and B; compound C; and compounds D and E (Table 3.1). This grouping corresponds well with their substitution patterns and spectral properties (Collins et al. 1981).

Table 3.1: Histochemical reaction of phenolic acids and flavonol glucosides

Compound	Reagent				
	none	amino borinate (0.1%)	$AlCl_3$ (15%)	$FeCl_3$ (3%)	NaOH (0.01N)
Ferulic acid	blue	blue	white	blue	blue
p-Coumaric acid	blue	blue	white	blue	yellow
Flavonol glucoside[1]					
A	yellow	yellow	yellow	--	--
B	yellow	yellow	yellow	--	--
C	yellow	yellow	--	--	--
D	white	white	white	--	--
E	white	white	white	--	--

[1] refer to their substitution pattern described in Fig. 1.1.

For ultrastructural studies, small segments of intact or acetone-treated leaves (see section (3.5)) were infiltrated with 1% aqueous caffeine solution for one hour at room temperature (Vaughn et al. 1980). The tissue was further sliced into strips of about 1 mm in the fixative solution. Fixation was carried out overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, followed by postfixation in 1% OsO_4 in the same buffer. After rinsing in the buffer and dehydrating in a series of ethanol, the material was embedded in Quetol (Bullock and Petrusz 1982). Ultrathin sections were mounted on nickel grids coated with formvar. Sections were stained with uranyl acetate followed by lead citrate before being examined with the electron microscope (Siemens Elmiskop). Control sections were submitted to the same fixation procedure except for the omission of the caffein treatment.

3.3.2. Alkaloids

Various histochemicals were used in an attempt to localize vindoline in leaf tissue. The nitrous reagent was prepared as described by Pearse (1980), and those of Dragendorff and Jeffrey, as well as ceric ammonium sulfate, were prepared following the protocols of Yoder and Mahlberg (1976). In the present study, the iodine stain (James 1950) was sligthly modified by acidification to pH 2 and alkalinisation to pH 10. Colour reactions of pure vindoline and vinblastine with these indicators were used as standards. Both alkaloids were obtained from the Plant Biotechnology

Institute. In this study, the plant material was incubated in the dark for five minutes with either of these reagents, thoroughly rinsed with PO_4 buffer (pH 7.2) and then observed with a bright field microscope.

3.4. Immunolocalization

3.4.1. Antibody production

Antiflavonoid antibody was previously prepared (Lamoureux et al. 1986) against 5,2',5',-trihydroxy-3,7,4'-trimethoxyflavone-2'-O-glucoside (flavonol A), the major flavonoid constituent of C. americanum. This latter was conjugated to bovine serum albumin (BSA) by the diazo reaction before being injected in the rabbit. The characterization of antiflavonoid antiserum was carried out by counter-electrophoresis and ELISA (Lamoureux et al. 1986). It has been shown that antibodies exhibited high specificity for flavonols that are 2'-O-glucosylated (compounds A and B, Figure 1.1).

Antivindoline antibody was kindly supplied by Dr. V. De Luca. In order to obtain an immunogenic compound, desacetyl vindoline and glutaric anhydride were reacted in pyridine to produce the glutarate hemiester of vindoline. The latter was coupled to bovine serum globulin (BSG) as described by Westekemper et al. (1980). The characteristics of the antiserum were found to be very similar to those obtained by Westekemper et al. (1980). Maximum affinity constants of the antibodies were 1.35×10^{-9} mol/L, revealing the presence of high affinity antibodies in the serum. Using an antibody

dilution of 1:150, the lowest detectable amount of vindoline was 2.42 pmol. Specificity studies of the antiserum were performed as described by Westekemper et al. (1980). No cross reactivity was observed with other alkaloids such as catharanthine, tabersonine, ajmalicine, serpentine or vinblastine. There was some cross reactivity (% of vindoline) with deacetyl vindoline (10.5), dihydrovindoline (7.2) and vindorosine (2.3).

3.4.2. Tissue preparation for light microscopy

Due to solubility of the antigen (flavonol or vindoline) in dehydrating agents normally used for permanent preparations, fresh or frozen material was utilized in this investigation. Epidermal strips of C. americanum were prepared by peeling off the lower epidermis from young and mature leaves, and special care was taken to avoid contamination with mesophyll tissue. For thin cross sections of leaves, segments of ca. 3mm were embedded in 20% (w/v) gelatin, rapidly frozen in CO_2 and then sectioned (8 um) at -30^0C using a Slee International cryostat. Thick cross sections (about 1mm) were hand-cut from young and mature leaves of both plant species. Immunofluorescence labeling of protoplasts was achieved in situ following their fixation in 1% glutaraldehyde in their respective osmoticum (see section 3.2). This fixation procedure is essential in order to preserve the integrity of protoplasts during their subsequent purification and immunolabeling.

3.4.3. Tissue preparation for electron microscopy

3.4.3.1. Chrysosplenium americanum

Immunocytochemical localization of the polymethylated flavonol A was performed by a post-embedding technique of samples taken from intact and in vitro cultured tissues. Small segments of leaves, calli and apices from in vitro regenerated shoots were sliced into 1-2 mm strips in the fixative. Fixation was carried out for 1 h in 3% glutaraldehyde (in 0.1M cacodylate buffer pH 7.2), followed by postfixation in 1% OsO_4 (in the same buffer) for 30 min. After rinsing in cacodylate buffer and dehydrating in a series of EtOH, the tissue was embedded in Epon. Ultrathin sections (90 nm) were mounted on 200 mesh nickel grids coated with Formvar.

3.4.3.2. Catharanthus roseus

The immunocytological localization of vindoline was performed with a suspension of protoplasts (pre-embedding technique) and on embedded sections of protoplasts and leaf segments (post-embedding technique). Prior to their purification, protoplasts were fixed in 1% glutaraldehyde in 3mM Mes buffer (pH 6.0) containing 0.7 M sorbitol and 3 mM $CaCl_2$ (the osmoticum). The same fixative, but diluted in PBS buffer, was used for leaf segments. Other fixatives and fixation procedures were tried in order to prevent the diffusion of vindoline during tissue preparation. These included 2% of 1-ethyl-3(3-dimethyl-amino-propyl)carbodiimide (EDC), 1% ceric ammonium sulfate (CAS) or 8% bismuth

subnitrate (BSN). Fixation with EDC was performed as described by Skene et al. (1987) and those with CAS and BSN followed a standard protocol (Yoder and Mahlbergh 1976). The effects of these fixatives on the cellular integrity were evaluated by light microscopy. ELISA and chromatographic procedures were also used to assess the effect of these fixatives on the immunogenicity of vindoline antibody and vindoline leaching, respectively.

Fixed protoplasts were purified and embedded in 1% agarose (Seed 1980) which was diluted in the osmoticum. Protoplasts and leaf segments were dehydrated in a series of EtOH and embedded in Lowicryl K4M or in Epon. Alternatively, protoplasts were protected with 20% glycerol (in the osmoticum) before being cryofixed in liquid propane at $-185^{0}C$ using a Reichert-Jung KF80 system (Vienna, Austria). In this case, protoplasts were transferred to liquid nitrogen, cryosubstituted (Reichert-Jung CFC auto cryosubstitution system) in 100% anhydric EtOH at $-90^{0}C$ and finally embedded in Lowicryl K4M according to the protocol of Carlerman et al. (1982).

3.4.4. Tissue labeling

3.4.4.1. For immunofluorescence

Plant tisssues are usually rich in various moieties with nonantigenic affinity for immunoglobulins. These interfering charges on leaf sections and protoplasts were blocked with 1% (w/v) gelatin in PBS (or osmoticum) for 15 min.

Immunofluorescence labeling was performed by the indirect method (Fig. 2.1). The primary antibody binding was carried out in a moisture chamber at 20^0C for 1 h. For this incubation, a dilution of 1:60 and 1:30 was respectively used for antiflavonol and antivindoline antisera. For protoplasts, the infiltration of the primary antibody was achieved under reduced pressure for 5 min with or without phosphatidylcholine (0.1%), oleic acid (0.1%) or Triton X-100 (0.01 to 0.1%). Following the antibody binding, samples were rinsed thrice, each for 3 min, then incubated with a fluorescent (fluorescein isothiocyanate) secondary antibody for 15-30 min. The goat antiserum was diluted (1:60) in order to give specific labeling with a low background level. Finally, after three more rinses in PBS and one in distilled water, the material was mounted in 50% glycerol containing 1 mg/ml of m-phenylenediamine, an anti-quenching reagent. The labeled material was then observed with an epifluorescence microscope equipped with FITC filter No. 9 (exciter filter, A450-490nm; dichroic mirror, A510nm; barrier filter, A520nm). Ektachrome 400 or 800-1600 day-light and Kodak Tri-X Pan 400 were used for color and black and white prints, respectively. The time of exposure was varied between 0.5 and 5 min depending on the intensity of fluorescence.

3.4.4.2. For immunocytochemistry

Ultrathin sections (gold interference color) were cut from embedded material and placed on 200-mesh nickel grids coated with Formvar. The grids were washed with distilled

water and floated for 15 min on a droplet of "Blotto" (5% w/v) (Johnson et al. 1984) and for 1 h on sodium borohydrate in order to block interfering aldehyde groups. Samples were then processed for labeling as described in the Immunofluorescence section, except that the secondary antibody was conjugated to colloidal gold (10 nm) after 25-fold dilution. Sections were stained with 2% aqueous uranyl acetate followed by lead citrate before being observed in a JEOL 1200EX electron microscope.

3.4.4.3. Specificity of labeling

The specificity of the immunolabeling was assessed using the following controls: (a) adsorption of the antibody by its respective antigen (flavonol A or vindoline), (b) using nonimmune serum instead of the specific antiserum and (c) using the secondary antibody alone.

3.5. Biochemical analysis

Various organs and tissues of C. americanum were analysed for their qualitative and quantitative content of flavonoids. Leaf effusate was obtained by repeated dipping for 2-3 seconds in either acetone or benzene-ethylacetate (1:1) over a period of 2 minutes. Epidermal strips were peeled off the lower surface of the leaf, while avoiding contamination with mesophyll tissue. Polymethylated flavonol glucosides of Chrysosplenium were extracted thrice from plant tissues with hot 80% methanol. The extracts were concentrated and chromatographed either on TLC as previously described (Collins

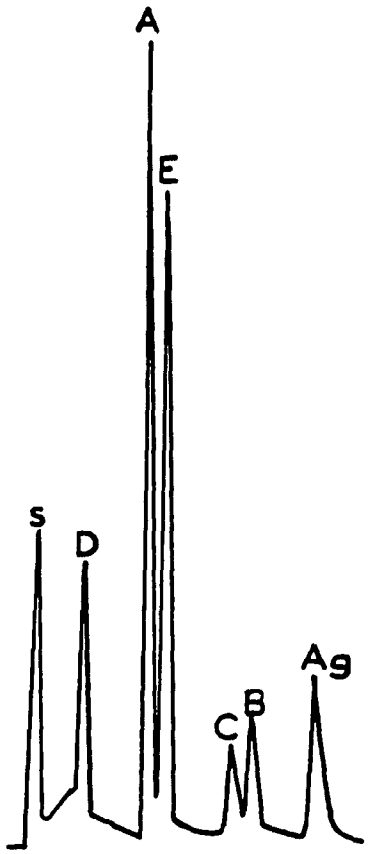


Figure 3.1. HPLC elution profile of the partially O-methylated flavonol glucosides (PMFGs) (A - E) as listed in Fig. 1.1, and aglycones (Ag); s: solvent.

et al. 1980) or by HPLC. In the latter case, the methanolic extract was passed through a C-18 Sep-Pak cartridge (Waters Associates) before being injected into the column. HPLC of the flavonoid glucosides was performed on a Waters-Millipore solvent delivery system using a C-18 Novapack column, a 340 nm filter and a linear gradient. The latter was composed of solvent mixtures: A, methanol-water-acetic acid (90:10:0.5) and B, methanol-water-acetic acid (20:80:0.5) and was run at a flow rate of 1ml/min. Identification of flavonoids was based on co-chromatography with authentic samples and their retention times by HPLC (Fig. 3.1).

Alkaloids of intact leaves of Catharanthus were extracted thrice with hot 80% methanol. The methanolic extracts were concentrated and chromatographed on TLC plates as described previously (DeLuca et al. 1986).

4. RESULTS.

4.1. Histochemical localization

4.1.1. Flavonoids of Chrysosplenium

Chrysosplenium leaves exhibit simple cellular organization, which consists of three to four layers of highly vacuolated mesophyll cells, bordered by two epidermal layers (Fig. 4.1a). Various cells of leaf tissues fluoresce following excitation with UV light. A strong blue fluorescence was observed in the walls of vascular cells and in the vacuoles of guard cells (Table 4.1). Moreover, a faint yellow fluorescence was observed in the walls of most epidermal cells. Regardless of the cell type, fluorescence appeared more intense in mature than in young leaves (Table 4.1). Whereas the vacuoles of vascular and guard cells fluoresced following a treatment with ferric chloride, epidermal cell walls lost their characteristic fluorescence. According to the histochemical reactions obtained with standard compounds, these observations seem to indicate that vascular and guard cells are rich in phenylpropanoids whereas epidermal cells were abundant in flavonoids. This is further substantiated by HPLC analysis of Chrysosplenium leaves (Table 4.2), demonstrating the recovery of flavonol glucosides in the epidermis as well as in the effusates. The latter which was prepared by dipping leaves in organic solvent suggested an external accumulation of these compounds. However, figure 4.1b shows the apical region of C. americanum observed in the

Figure 4.1. Leaf of Chrysosplenium americanum and Catharanthus roseus:

A) cross section of Chrysosplenium observed in bright field microscope (200X),

B) apical leaves of Chrysosplenium observed in scanning electron microscope (125X),

C) cross section of Catharanthus stained with iodine. Cells reacting with iodine are referred as idioblasts (150X).

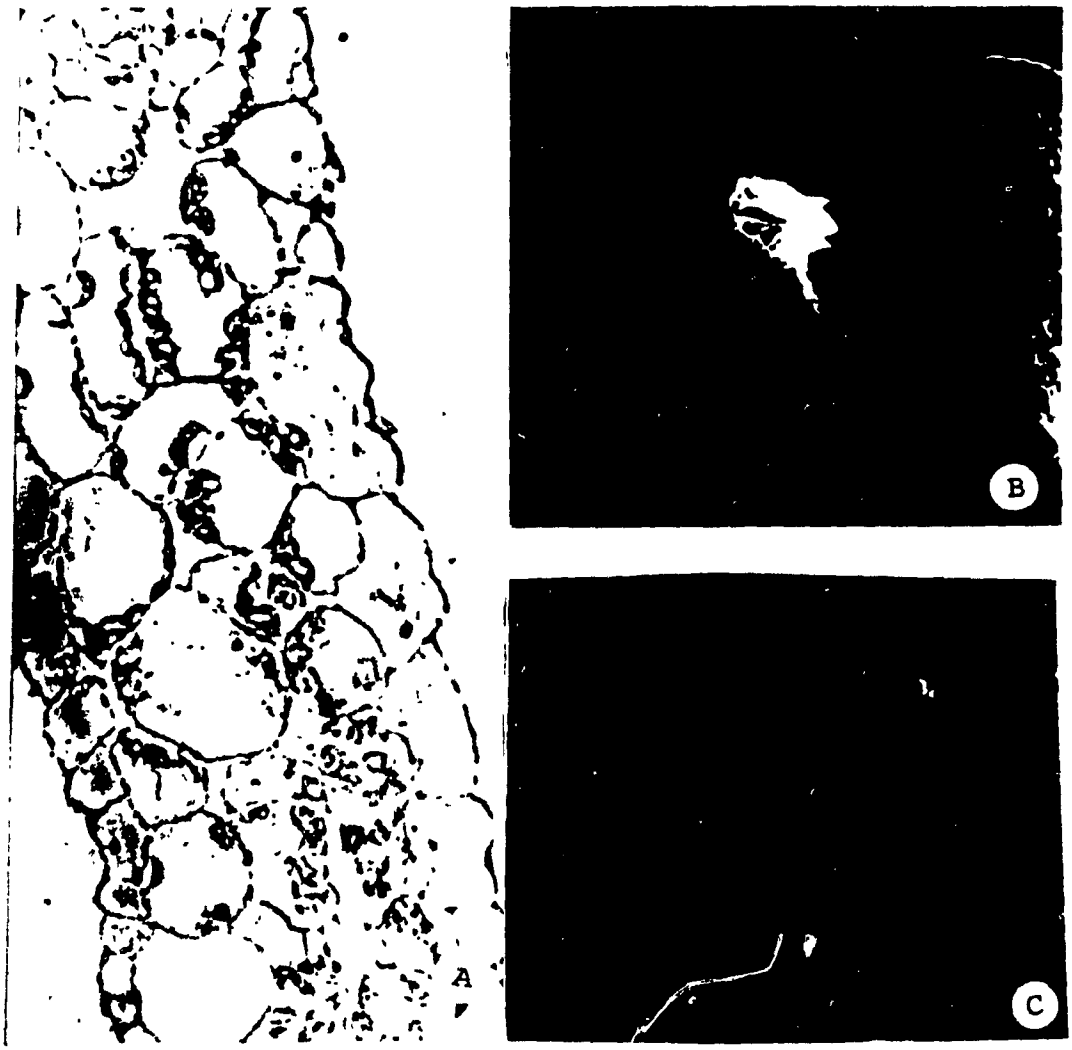

A
B
C

Table 4.1. Fluorescence of epidermal cells from apical and mature leaves of *Chrysosplenium americanum*

Leaf Position	Colour in	Colour with			
	UV light	amino borinate	$AlCl_3$	$FeCl_3$	NaOH
Apical leaf					
Guard cell					
vacuole	blue	+	-	yellow	white
cell wall	blue	+	blue	blue	blue
Epidermal cell					
vacuole	-	-	-	-	-
cell wall	-	yellow	yellow	-	-
Mature leaf					
Guard cell					
vacuole	blue	+	-	blue	-
cell wall	blue	+	blue	blue	blue
Epidermal cell					
vacuole	pale blue	+	-	-	-
cell wall	yellow	+	yellow	-	yellow

-: no reaction

+; denotes an intensification of the natural autofluorescence

Table 4.2. Flavonoid composition of Chrysosplenium americanum

Tissue	Flavonoid composition [1]					
	Glucosides [2]					Agly-cones
	A	B	C	D	E	
a) Intact leaf						
Apical	38.0	9.7	6.3	12.5	33.5	n.d.
Lower	32.9	7.2	6.7	13.2	33.7	n.d.
b) Epidermal layer						
Apical leaf	36.8	8.6	5.3	11.4	37.7	n.d.
Lower leaf	37.1	6.2	5.1	9.7	32.5	9.4
c) Leaf effusates						
Acetone extract						
Apical leaf	35.2	8.2	4.4	11.5	36.3	4.2
Lower leaf	31.1	6.7	4.8	11.6	32.4	13.4
Benzene-ethyl acetate extract						
Apical leaf	8.8	2.5	2.2	0.4	4.0	82.1
Lower leaf	8.2	2.1	2.2	1.3	6.6	79.6

[1] % of total as determined by HPLC and integration of peak areas.
[2] Glucosides: A, 5,2′,5′-trihydroxy-3,7,4′-trimethoxyflavone-2′-O-glucoside; B, 5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone-2′-O-glucoside; C, 5,5′-dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-O-glucoside; D, 5,2′,5′-trihydroxy-3,6,7,4′-tetramethoxy-flavone-5′-O-glucoside; E, 5,5′-dihydroxyflavone-3,6,7,2′,4′-pentamethoxy-flavone-5′-O-glucoside.
n.d., not determined.

scanning electron microscope. The surface of leaves and stem strongly suggests that this plant material does not excrete any lipophilic substances.

4.1.2. Alkaloids of Catharanthus

Catharanthus leaves exhibit a typical C-3 cellular organization with distinct palisade and spongy mesophyll tissue (Fig. 4.1c). Of all the alkaloid detecting reagents used, only iodine in potassium iodide reacted positively with certain cells of Catharanthus without causing any noticeable cell damage. The loss of cellular integrity caused by BS and CAS have restrained further histochemical use of these reagents. Cells reacting with iodine were randomly distributed within the mesophyll and more concentrated in the vicinity of vascular tissues (Fig.4.1c). These cells can be distinguished from other mesophyll cells by their large size, low plastid content and, their fluorescence properties which were similar to the idioblasts and laticifers previously described by Mersey and Cutler (1976). However, no differences were observed in relation to their frequency and distribution among various leaves of different level of maturity.

4.2. Ultrastructural features of Chrysosplenium americanum

4.2.1 Cultured cells

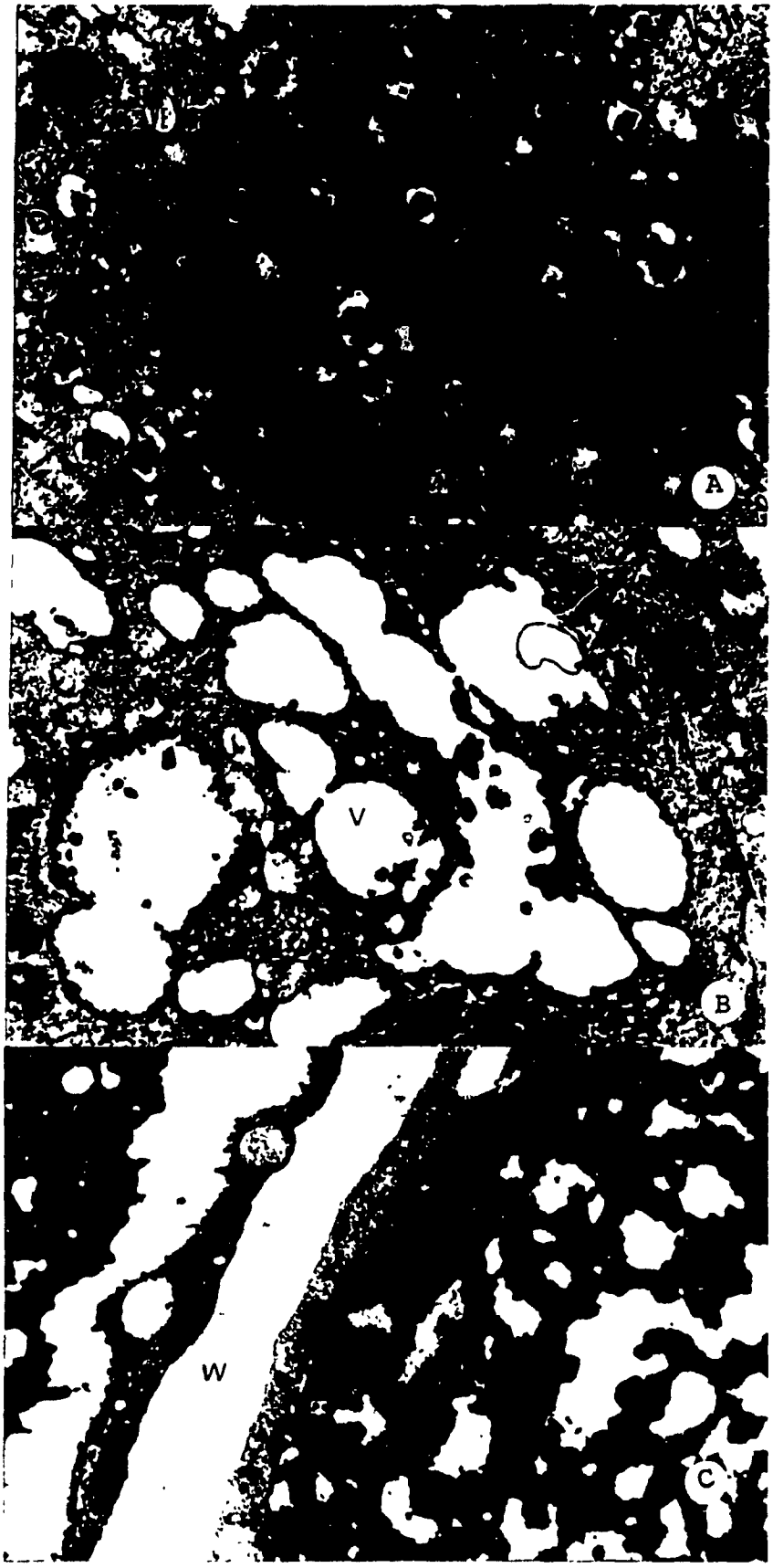
Figures 4.2a and 4.2b show the similarities in the ultrastructural features of the intact leaf tissue and an in vitro cultured explant (see Appendix). The ultrastructure of the various cell types appeared well preserved as indicated

Figure 4.2. Ultrastructural features of Chrysosplenium cell from intact plant (A; 45 000X) and in vitro explant (B; 37 000X). In both cells, a similar cellular organisation is observed in term of the number and appearance of organelles such as: cell wall (W), chloroplast (C), mitochondria (M) and vacuoles (V). However, cell cultured in vitro contains an extensive endoplasmic reticulum (R).


V
A
W
C
V

Figure 4.3. Variety of cell types observed in an explant of *Chrysosplenium* cultivated *in vitro* (8 000X).

Figure 4.4. Electron dense deposits observed in vesicles (VE), (A: 10 000X); vacuoles (V) (B: 10 000X) and cytosol (C: 15 000X) of Chrysosplenium cells. Note that an intense accumulation of these deposits (tannins) seems to cause cell damage.

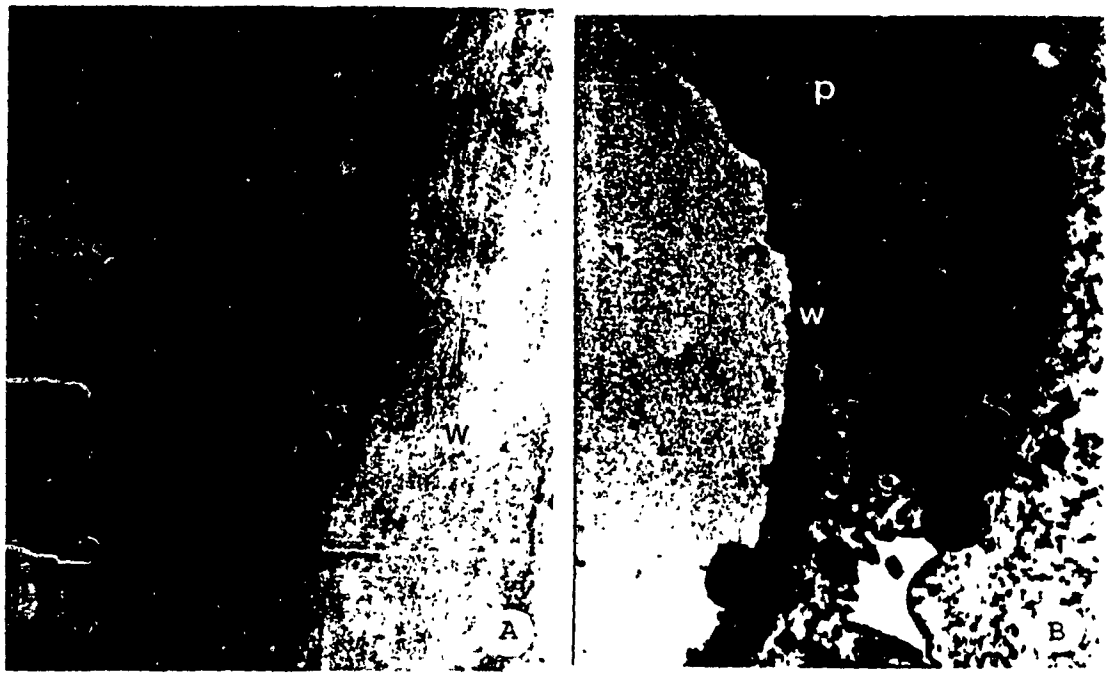

A
V
B
W
C

by the integrity of organelles and membranes. However, the ultrastructural study of cultured tissues allows a fast screening of various cells (Fig. 4.3). Among them, some are noticeable by the presence of electron dense material deposited in the lumen of vesicles and vacuoles, and are closely associated with the tonoplast (Fig. 4.4). In addition to these vesicles, these cells are also characterized by extensive undulation of their plasmalemma, which are suggestive of their secretory nature, or an intense metabolic activity. Concomitant with these observations, similar electron dense material seems to fill the entire cytoplasm of few cells (Fig. 4.4c), that appeared to be devoid of any organelles as well as endomembranous system (Fig. 4.4c). This suggests that small vesicles coalesce and form larger vacuoles and finally, fill the entire cell. These features were similar to the tannin-cells previously described by Mueller and Beckman (1974; 1976).

4.2.2. Intact leaves treated with caffeine

Caffeine treatment of Chrysosplenium leaf segments, prior to fixation, did not cause any alteration in the ultrastructure of its cells (Fig. 4.5). However, compared to control cells, caffeine caused an opacity of different cell compartments and particularly the cell wall (Fig. 4.5b and 4.5c). Various membrane profiles and vesicular structures appeared more electron dense when they were prefixed with caffeine (Fig. 4.5b and 4.5c). In addition, the intracellular spaces of the latter cells contained similar electron dense

Figure 4.5. Caffeine treatment of Chrysosplenium leaves:
A: control leaf (25 000X),
B & C: caffeine treated leaves (B: 10 000X & C: 50 000X).
The darkening, indicative of a positive reaction with caffeine, is intense in cell wall (W), plasmalemma (P) and in the membrane of vesicle (VE).


W
A
p
w
B


ve
C

material. On the other hand, the Golgi apparatus, chloroplasts, mitochondria and nuclei appeared translucent (Fig. 4.5e). However, electron-dense material was often observed in association with the tonoplast. These characteristic features appeared in both the adaxial and abaxial layers of the leaf epidermis as well as mesophyll cells. All cells containing electron-dense deposit appeared to have dense cytoplasm and intact organelles. However, the localized deposits on mesophyll cell walls were less frequent than those on the epidermis.

4.3. Immunofluorescence

4.3.1. Flavonoids of Chrysosplenium

Preliminary work using different fixative and dehydrating solvents to immunolocalize flavonoids gave unsatisfactory results due to tissue damage and leaching of intracellular flavonoids. I resorted, therefore, to the use of frozen leaf sections and epidermal strips as well as protoplast suspensions. The yellow-green color of fluorescein, indicative of the presence of Chrysosplenium flavonoid glucosides, could be easily distinguished from the yellow autofluorescence of cell walls, the red fluorescence of chloroplasts and the blue fluorescence in the vacuoles of guard cells. Figure 4.6 shows the specific immunofluorescence observed in the walls of epidermal and mesophyll cells. In both cell types, the fluorescence appeared unevenly localized in the cell walls and seemed to increase with the maturity of the leaves. The

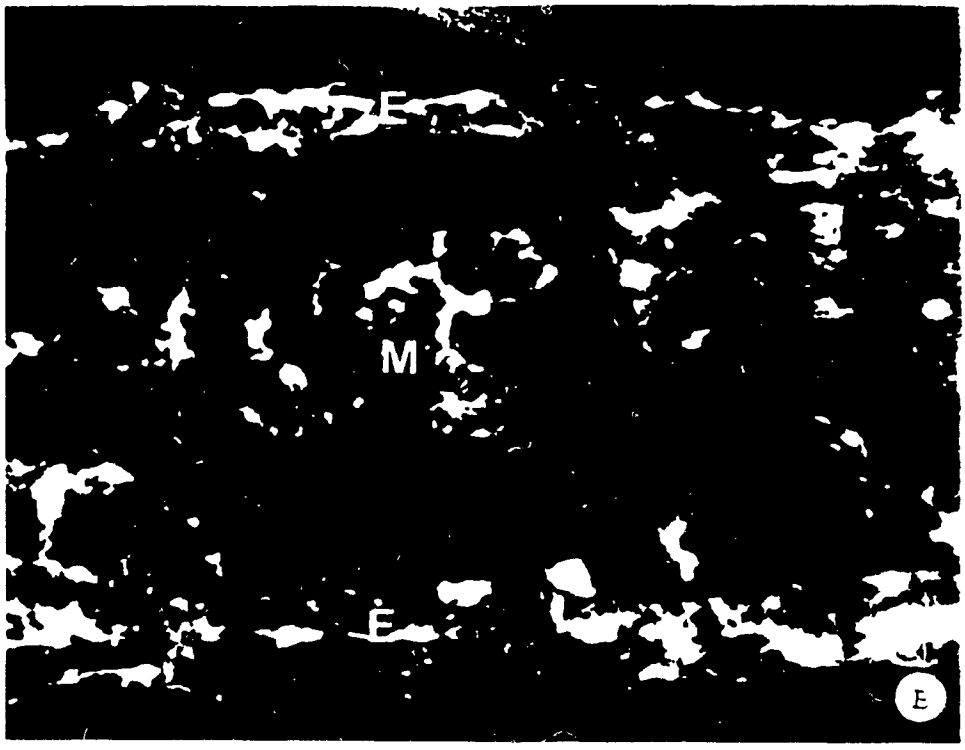
Figure 4.6. Immunofluorescence labeling of PMFGs in a leaf cross section of C. americanum (3 min exposure) incubated first with:

A) Control serum (300X);

B) Anti-flavonoid serum (300X).

The specific immunofluorescence is observed in the walls of epidermal cells (E) and to a lesser extent in the walls of mesophyll cells (M).


E
M
E
A


E
M
E
B

observation of intact epidermal layers further confirmed the association of fluorescein with the walls of these cells (Fig. 4.7).

There was no fluorescence observed in the vacuoles of either epidermal or mesophyll cells of leaf sections. This may have been due to possible leaching of cellular constituents during tissue processing. However, in situ labeling of mesophyll protoplasts revealed a faint immunofluorescence in their vacuoles (Fig. 4.8) which required a long time of exposure to be recorded on the film. However, this vacuolar fluorescence was observed in a small proportion of the protoplasts examined (about 10%). The absence of blue emission following treatment with calcofluor, a cell wall indicator, suggested that this fluorescence was not due to the presence of residual or regenerating cell walls.

4.3.2. Vindoline of Catharanthus

As mentioned previously, the alkaloid precipitating reagents BS and CAS resulted in serious cell damage and were not used in the immunological studies. On the other hand, EDC seemed to preserve cellular integrity although it did not prevent the leaching of vindoline during tissue preparation. Since no chemical reagent is known to fix vindoline without damaging the cells, its detection by immunofluorescence was performed on fresh, hand-cut leaf sections and with protoplast suspensions. In this study, the yellow-green epifluorescence of fluorescein, indicative of the presence of vindoline, was easily distinguished from the yellow and red autofluorescence

Figure 4.7. Immunofluorescence labeling of PMFGs in the lower epidermis of C. americanum (1 min exposure) incubated first with:
A) control serum (250X);
B) anti-flavonoid serum (250X).
Observe the autofluorescence in stomata (S) and the specific immunofluorescence in the cell wall (W).

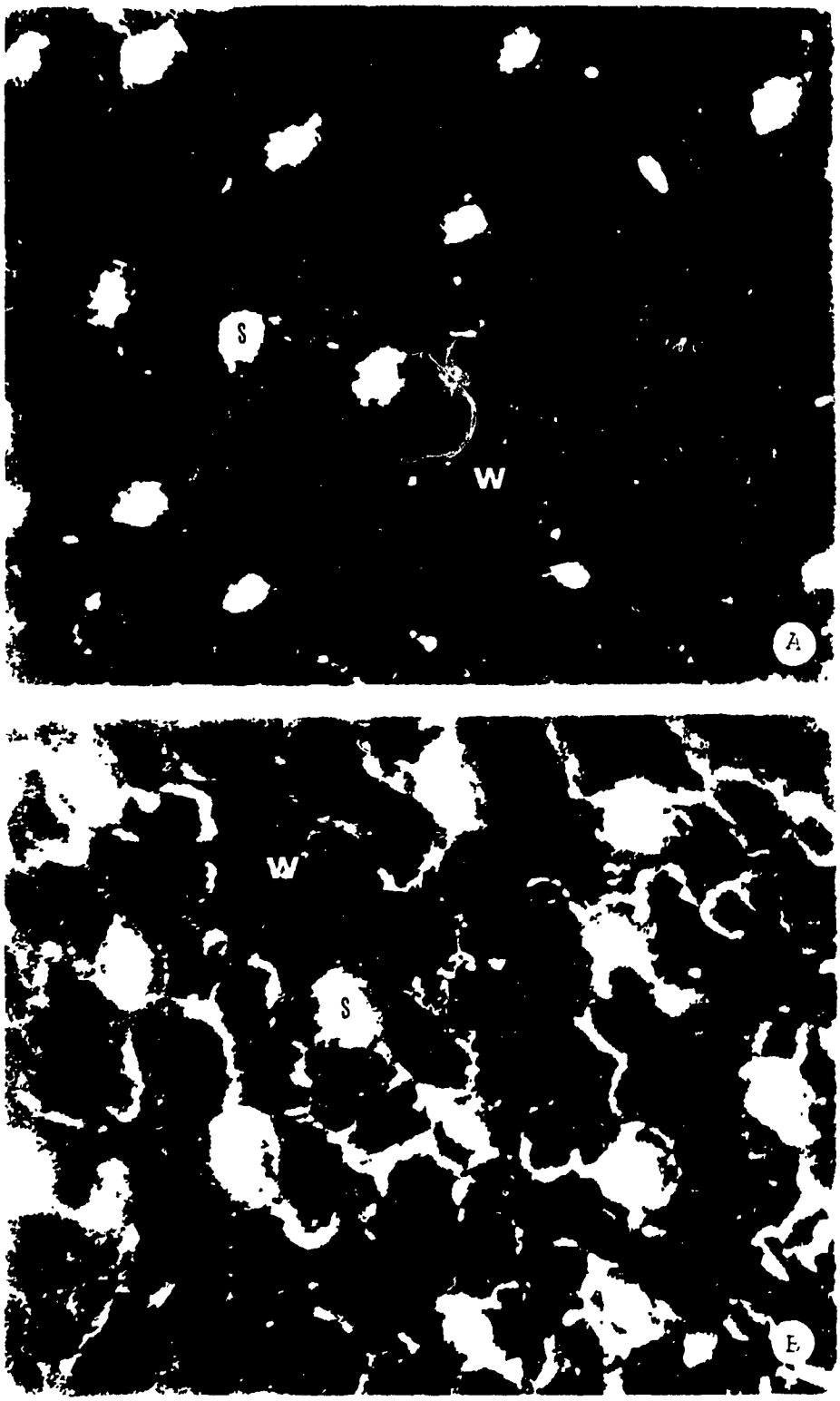
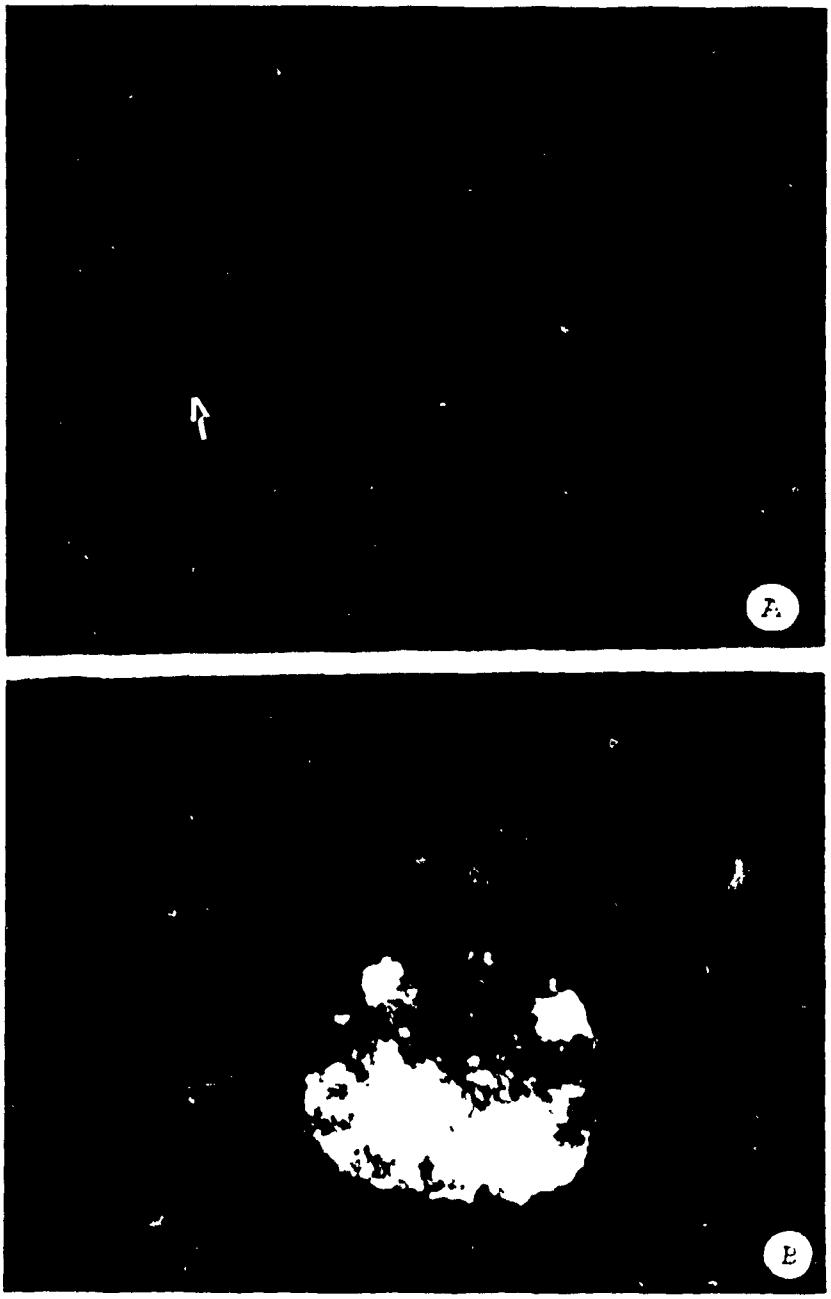

S
W
A
W
S
B

Figure 4.8. Immunofluorescence labeling of PMFGs in leaf mesophyll protoplasts of C. americanum (5 min exposure) incubated first with:

A) control serum (500X), arrow indicates position of protoplast;

B) anti-flavonoid serum (500X).


A
B

observed in idioblast vacuoles and plastids, respectively. Figure 4.9 shows the association of antibody labeling in subepidermal and many mesophyll cells. The autofluorescence properties of the reactive subepidermal cells seem to correspond with those described for laticifers by Yoder and Mahlberg (1976). In mesophyll tissue, vindoline was not only found in idioblasts, but was also detected in the vacuoles of many non-specialized parenchyma cells (Fig. 4.9). However, the inconsistency among the replicates examined, as well as the disperse labeling of fluorescein may be attributed to the diffusion of vindoline from idioblasts to the surrounding cells. Therefore, evidence for the accumulation of vindoline in the vacuoles of both idioblast and parenchyma cells was obtained from *in situ* immunolabeling of protoplast preparations (Fig. 4.10). A positive reaction was detected in all idioblasts and in most mesophyll protoplasts examined. In contrast with leaf cross sections, the immunofluorescence labeling of both cell types was consistent among all the replicates examined and therefore, strongly suggests the accumulation of vindoline in the vacuoles of both idioblasts and mesophyll protoplasts.

4.4. Immunocytochemistry

4.4.1. Flavonoids of *Chrysosplenium*

Both leaf tissues of intact and in vitro grown explants fixed with glutaraldehyde and OsO_4 showed good ultrastructural preservation and maintained the antigenicity of the hapten

Figure 4.9. Immunofluorescence labeling of vindoline in leaf cross section of Catharanthus roseus incubated first with:

A) control serum (300X);

B) anti-vindoline serum (300X).

The yellow-green fluorescence of fluorescein appears in subepidermal and mesophyll cells and, particularly in cells located in the vicinity of vascular tissue (V).


A


B

Figure 4.10. Immunofluorescence labeling of vindoline in vacuoles of leaf protoplasts of C. roseus incubated first with:

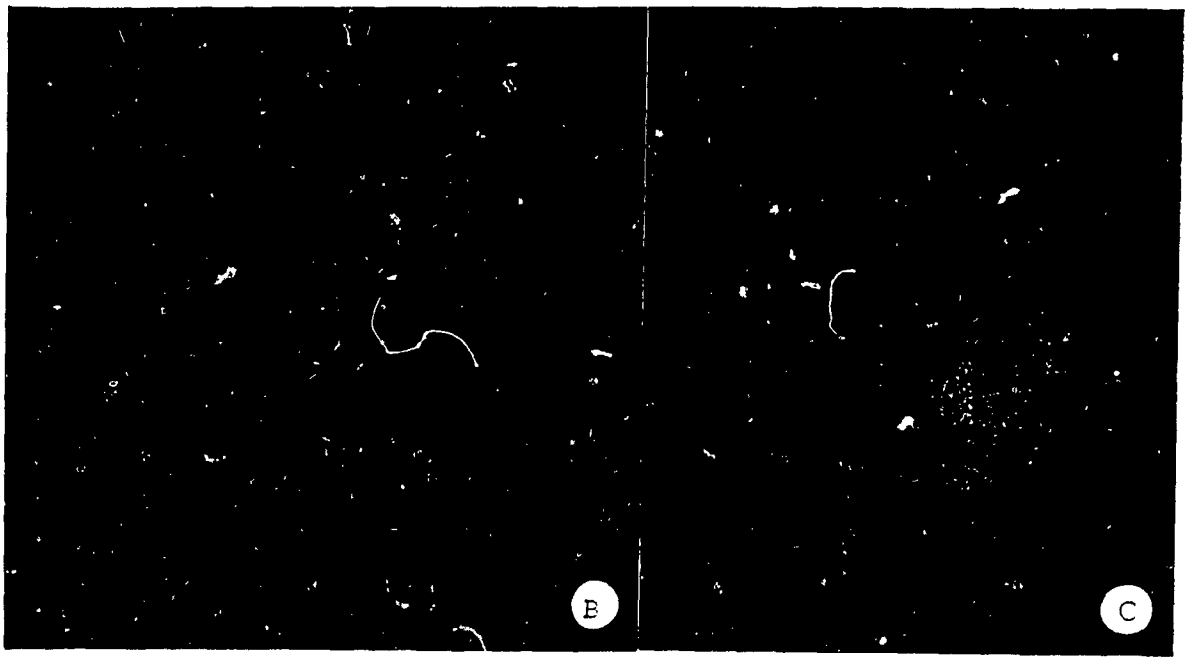
A) control serum (1 500X);

B and C) anti-vindoline serum;

B) mesophyll protoplast (1 500X),

C) idioblast (1 250X).


A


B
C

(Fig. 4.11; 4.12 and 4.13). Compared to control sections, the gold label was mainly localized in the cell wall and to a much lesser extent, in the vacuole. Walls of the epidermis were heavily labeled in the vicinity of the plasmalemma. Furthermore, labeling was also detected in the walls of mesophyll cells. Regardless of the cell type, no labeling was observed in vesicles, nuclei, Golgi apparatus, chloroplasts or mitochondria.

The immunocytochemical examination of *in vitro* cultured tissues allowed the study of the density of labeling among different cells. A predominant labeling was localized in walls of protoderm (Fig. 4.12a). Furthermore, the walls of many mesophyll cells appear to contain a high concentration of flavonoids. The latter seemed to be particularly concentrated in cells characterized by an undulated plasmalemma and rich in vesicular structures (Fig.4.12b, c, d). In contrast, no labeling was found in the cellular structures of meristematic cells nor in those cells containing tannins (Fig. 4.13). In addition, as observed in intact leaves, no gold particles were detected on vesicles, nuclei, Golgi apparatus, chloroplasts or mitochondria of any cells (Figs. 4.11 and 4.12).

4.4.2. Vindoline in *Catharanthus*

Although the ultrastructure of *C. roseus* cells was well preserved after fixation with glutaraldehyde and OsO_4, specific labeling of vindoline could not be achieved with thin leaf sections (Fig. 4.14). This was attributed to the leaching of antigen during dehydration of the material and/or loss of

Figure 4.11. Immunocytochemical labeling of PMFGs in the leaf of C. americanum: Sections incubated first with:

A) control serum (30 000X);

B) anti-flavonoid serum (33 000X).

Specific labeling is observed in cell wall (W). Cytosol, mitochondria (M), Golgi apparatus (G), vesicles (VE), plasmalemma (P) and endoplasmic reticulum (R) are not labeled.


A
W
P
M
G
B

Figure 4.12. Intense immunocytochemical labeling of PMFGs in cell walls of protodermal (A: 15 000X) and mesophyll (B: 45 000X, C: 40 000X & D: 30 000X) cells of C. americanum. Labeling in mesophyll is particularly intense in cells characterized by their extensive endoplasmic reticulum and numerous vesicles. Observe the absence of gold particles in chloroplast (C), cytosol, endoplasmic reticulum (R), Golgi apparatus (G), intracellular space (IS) and mitochondria (M).

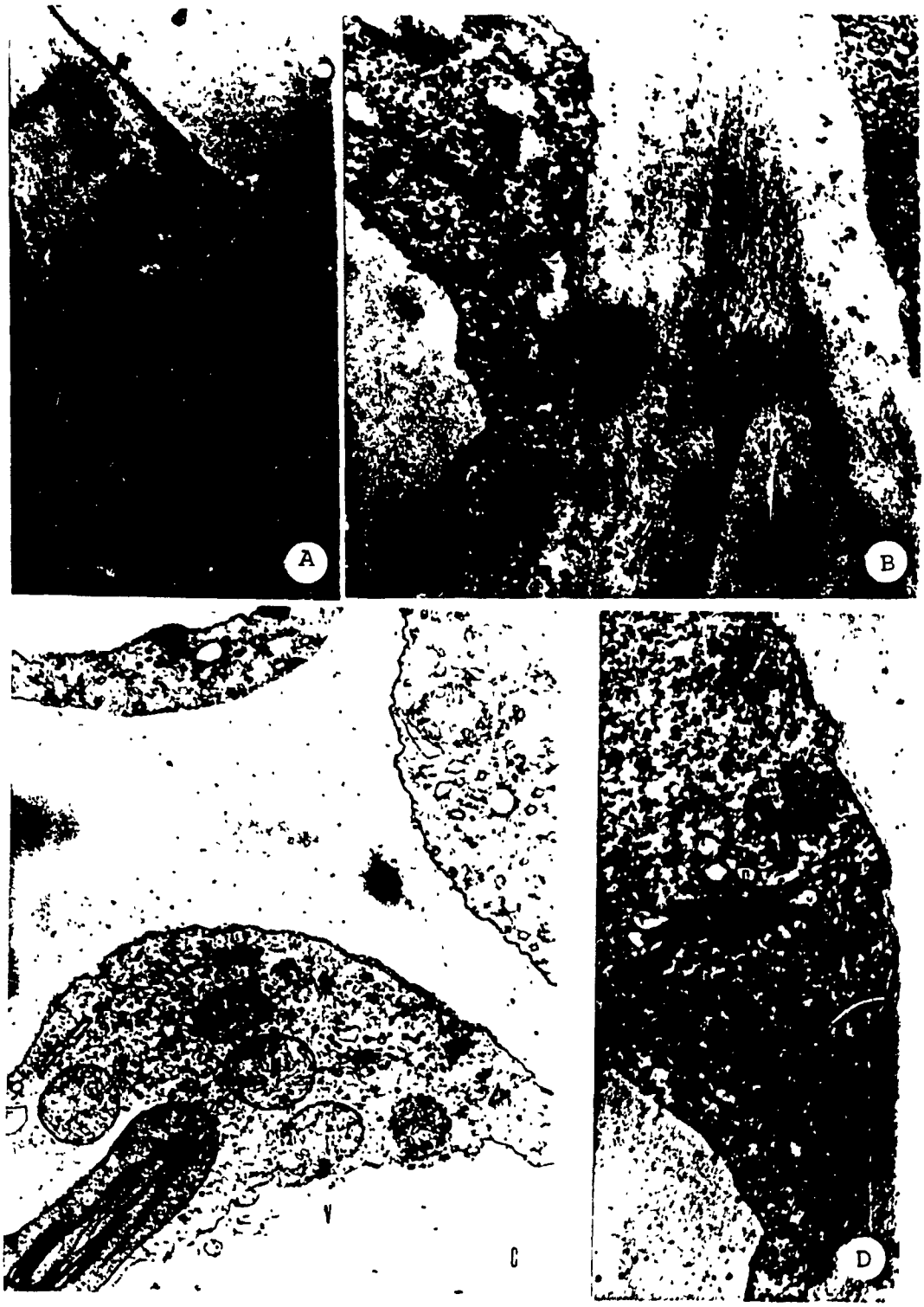
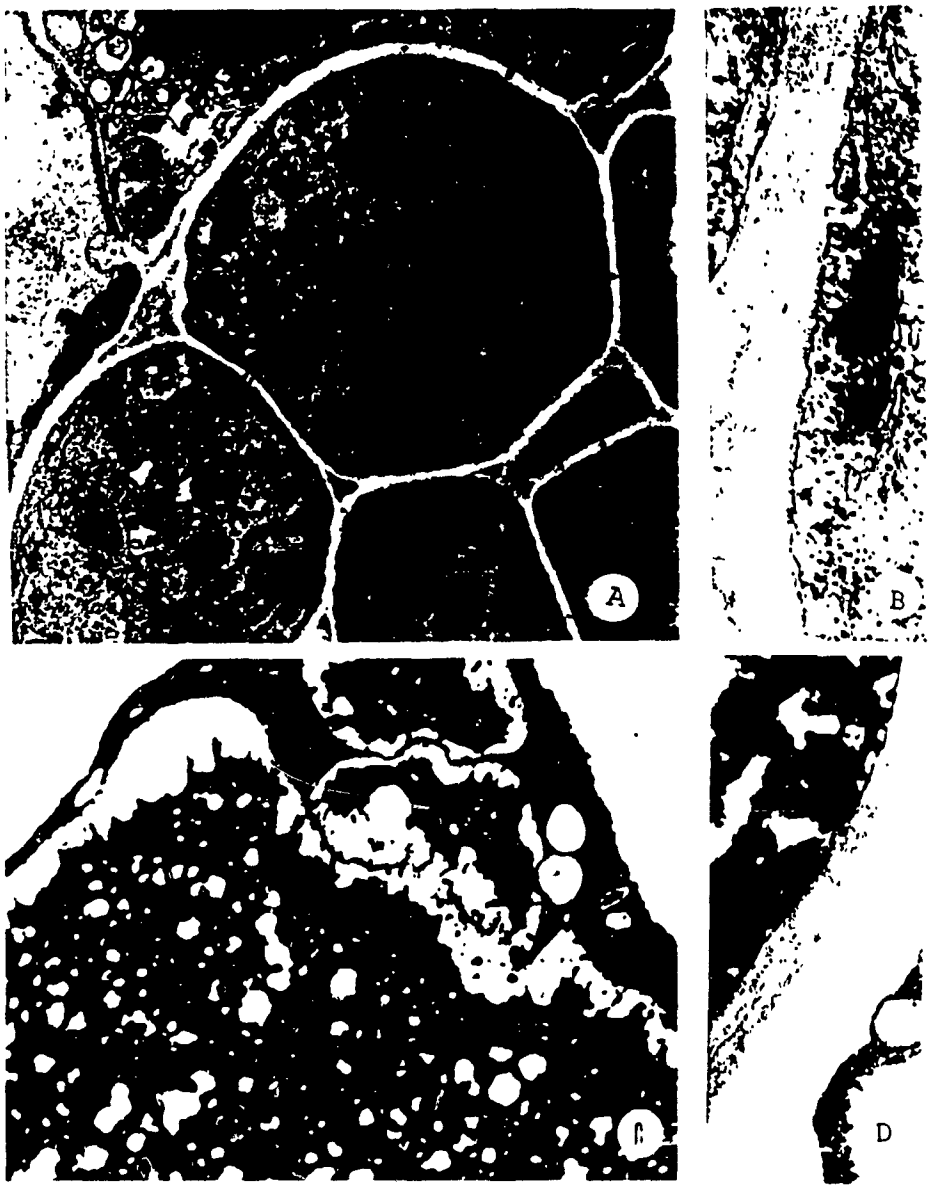

A
B
C
D

Figure 4.13. Sparse immunocytochemical labeling of PMFGs in meristematic cell (A: 6 500X), cell rich in tannins (C: 15 000X) and their respective cell walls (B: 25 000X & D: 15 000X).


A
B
C
D

antigenicity of the antibody. In fact, the inability of structural fixatives to retain vindoline was confirmed by chromatographic analysis. This led to the use of thin sections that were obtained from material prepared by means of cryotechniques. Such techniques allowed preservation of the intracellular distribution of vindoline, as well as to maintaining its antigenicity. However, the most significant problem encountered was the tendency for the cellular membranes to be poorly preserved. Figures 4.15 b-d present evidence that vindoline was predominantly localized in protoplast vacuoles and, to a lesser extent, in the cytoplasmic areas surrounding these vacuoles. Vindoline was also detected in chloroplasts, especially those in the vicinity of vindoline-containing vacuoles (Fig. 4.15c). In this study, it was not possible to collect enough material in order to establish the differential accumulation of vindoline in various cell types.

Figure 4.14. Ultrastructural features of Catharanthus roseus leaf cells. Leaf cells are characterized by numerous amyloplasts (A) (A: 7 500X, B: 16 000X & C: 45 000X). Observe the good preservation of membranes following conventional fixation with glutaraldehyde and OsO_4, (Chloroplast (C), mitochondria (M), plasmalemma (P) & tonoplast (T)).

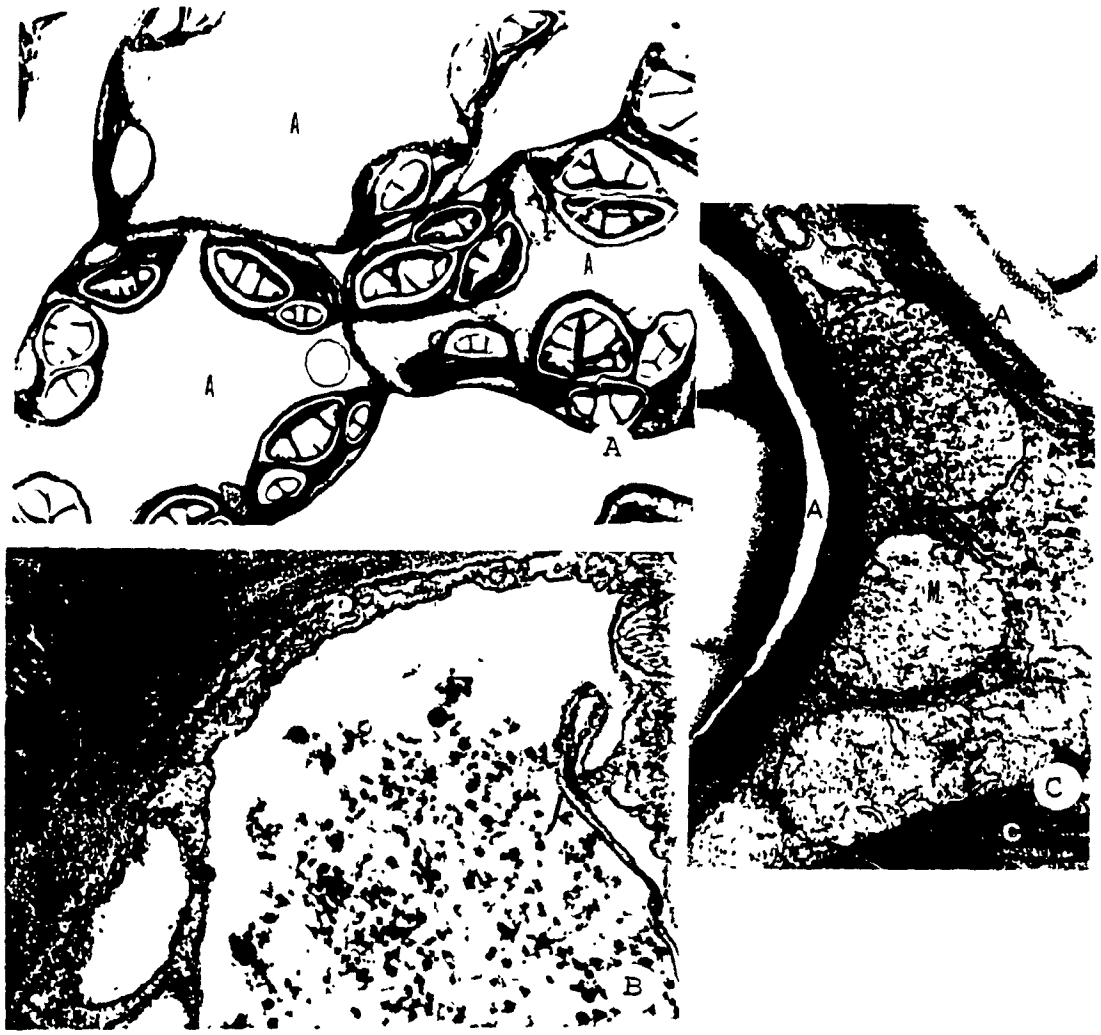
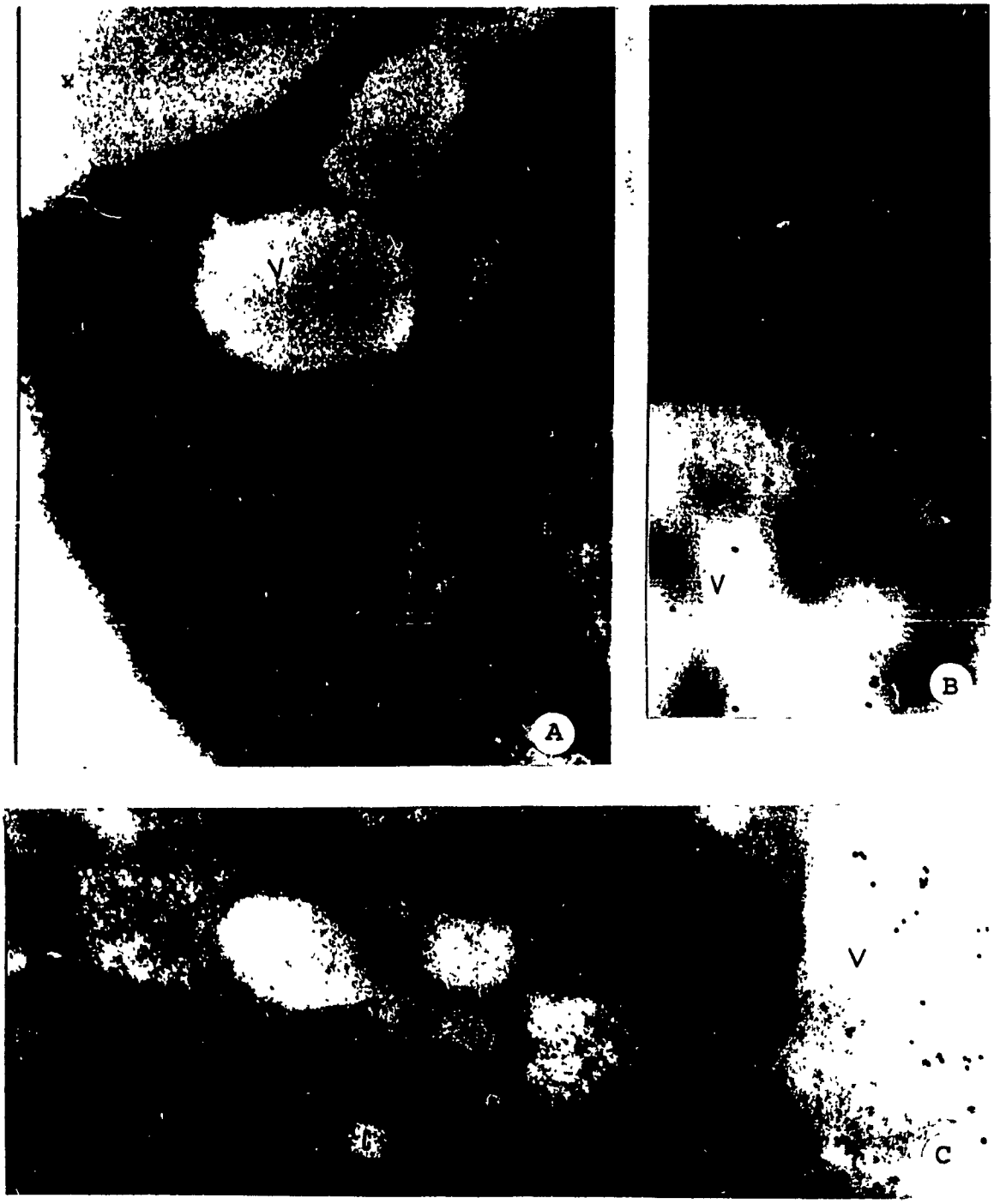

A
A
A
A
A
M
C
C
B

Figure 4.15. Immunocytochemical labeling of vindoline in cryofixed mesophyll protoplasts of Catharanthus leaves incubated first with:

A) anti-vindoline serum that was inactivated with vindoline (36 000X);

B and C) anti-vindoline serum (B: 69 000X; C: 36000X), (Chloroplast (C), mitochondria (M) & vacuole (V)).


V
A
V
B
V
C

5. DISCUSSION

5.1. Evaluation of immunological techniques

It is generally assumed that plant secondary metabolites are accumulated and stored in the cellular vacuole. However, in most cases, there has been little conclusive experimental evidence to support this assumption. This situation may arise from some technical difficulties related to the low concentration of plant secondary metabolites, their solubility in aqueous and organic solvents or the lack of specific reagents for their detection. Therefore, conventional localization studies using histo-cytochemistry, tracer experiments or cell fractionation techniques seldom provide unequivocal results. This situation compares with that of plant growth regulators, where their immunolocalization has recently contributed to the understanding of some important physiological events (Bertrand et al. 1989; Skene et al. 1987; Sossountzov et al. 1986; Sotta et al. 1985; Zavala and Brandon 1983).

In Chrysosplenium leaves, polymethylated flavonol glucosides were localized primarily in the walls of epidermal and mesophyll cells and to a lesser extent in the vacuoles. This has been confirmed using both immunofluorescence and immunocytochemical techniques as well as histochemical and chromatographic methods. Despite the fact that Chrysosplenium accumulates small amounts of tannins and phenylpropanoids, however, the latter did not interfere in the localization of

flavonoids due to the high specificity of antibodies raised against these metabolites.

Vindoline was localized mainly in the vacuoles of idioblasts, and mesophyll cells as well, although previous histochemical and chromatographic data reported the association of indole alkaloids with idioblasts, but not with mesophyll cells (Mersey and Cutler 1985; Neumann et al. 1983; Yoder and Mahlbergh 1976). Considering the high specificity of antibodies to vindoline, the detection of label in mesophyll cells reflects the sensitivity of immunological techniques.

Due to the solubility of most secondary metabolites in various solvents, their diffusion has restricted detailed localization studies. Fortunately, the association of Chrysosplenium flavonoids with the cell walls eliminated this problem. However, this situation contrasts with the difficulties encountered with the immunodetection of vindoline. The inconsistency between the replicates of leaf cross sections examined for immunofluorescence may reflect the leaching of vindoline from idioblasts, thus contaminating the surrounding mesophyll cells. Furthermore, the absence of specific gold labeling from sections prepared with conventional fixation and dehydration procedures also suggests the loss of vindoline during tissue preparation. Diffusional artifacts may be considerably reduced by observing hydrated material in light microscopy. Consequently, the retention of vindoline was first achieved by in vivo immunofluorescence

labeling of the protoplasts. Their permeabilization to sera and fluorescein was achieved by a transient exposure of protoplasts to hypotonic shock (Cutler and Saleem 1987) and an incubation under reduced pressure (Schachtele and Steup 1986). Furthermore, since the diffusion of vindoline through membranes was previously attributed to its low molecular weight, it seems reasonable to assume that its binding with antibodies would decrease subsequent leaching (Renaudin et al. 1985).

For EM studies, cryofixation and cryosubstitution procedures have been recommended for the ultrastructural localization of hydrophilic proteins and plant growth regulators. However, cellular damage caused by freezing and thawing of the plant material has frequently been encountered (Herman 1988). In addition, examination of micrographs published by several workers indicates that membranes were insufficiently preserved in the absence of osmium fixation (e.g. Baumgartner et al. 1980; Doman and Trelease 1985; Hosokawa and Ohashi 1988; McKay and Gibbs 1989). However, in this study, only cryofixed material of Catharanthus could retain vindoline with sufficient ultrastructural features to enable its unequivocal localization in the cellular vacuole. Nevertheless, the use of cryo-techniques represents a compromise between membrane preservation and retention of the metabolite under investigation (Herman 1988). Whereas the inadequate preservation of Catharanthus membranes may impede the interpretation of results, the accumulation of vindoline

predominantly in the vacuoles is strongly supported by the even distribution of gold particles in this compartment, as well as its localization by in vivo immunofluorescence. Therefore, the validity of these techniques can be attested for by the conformity of observations obtained at the tissue, cellular and subcellular levels.

Furthermore, these studies represent the first report that demonstrates the potential of immunological methods for the unequivocal localization of plant secondary metabolites. Compared to conventional methods, the immunodetection of such metabolites offers a number of advantages. First, this technique is quite reliable in view of its sensitivity and specificity of antibodies, as well as the specificity of labeling. In addition to proper identification of compounds, the label used (fluorescein or gold particles) is easily detectable. In general, the choice of using light (immunofluorescence) or electron (immunogold) microscopy depends on the level of resolution required. Due to the solubility of plant secondary metabolites, the use of both techniques is necessary for their immunodetection. In addition, simultaneous identification of the compound of interest and its sub-cellular compartment renders these methods superior to those based on either histo/cytochemistry or cell fractionation techniques. Nevertheless, future technical developments will be required to allow the fixation of soluble compounds while maintaining the ultrastructural integrity of organelles, as well as the antigenicity of the

molecule to be detected. We have shown that intact and cryofixed material may be used for immunolocalization studies in spite of the inadequate cryofixation of membranes. Alternatively, new fixation procedures such as the rapid fixation with microwave irradiation (Walsh et al. 1989) should be investigated. On the other hand, the recent development of tissue printing on nitrocellulose paper represents an interesting alternative to localize soluble compounds at the tissue level (Cassab and Varner 1987). Moreover, since the immunodetection of secondary metabolites is still in its "infancy", more experiments will be required to standardize the methodology and to ensure the validity of the techniques used.

5.2. Localization of flavonoids

In Chrysosplenium leaves, polymethylated flavonol glucosides were found to accumulate mainly in the epidermal layers, as has been reported for numerous flavonoids of most plant species (see Table 1.1). However, in contrast with the common occurrence of flavonoid glucosides in the vacuole, those of Chrysosplenium have been localized within the cell wall. Furthermore, this plant differs from other species which secrete their flavonoid aglycones on the leaf surface as farinose or gummy exudate (Bohm et al. 1986; Charriere-Ladreix 1973, 1975, 1976; Clark and Wollenweber 1984; Wollenweber 1984; Wollenweber and Dietz 1981). Thus, Chrysosplenium represents a unique example in view of the site of accumulation of its flavonoids, which is in agreement with

the solubility properties of these metabolites. The latter are mainly lipophilic due to the presence of three to five methoxyl groups on the flavonoid ring system, and are partly hydrophilic due to the presence of one glucosyl residue.

Numerous papers have attempted to correlate the site of accumulation of flavonoids with their ecological significance. The high concentration of flavonoids in the upper epidermis has been attributed to their ability to absorb UV irradiation (McClure 1975, 1979; Schmelzer et al. 1988; Tissut and Ravanel 1980; Weissenbock et al. 1987). On the other hand, Wollenweber has established a correlation between the secretion of flavonoid aglycones and the adaptation of the plant to a specific habitat such as arid or alpine conditions (Wollenweber et al. 1987). Similarly, the production and accumulation of polymethylated flavonol glucosides in the walls of epidermal cells may be correlated with the adaptation of Chrysosplenium to the semi-aquatic habitat. Therefore, these metabolites may constitute a chemical barrier in order to palliate the lack of lignified tissue and protect the plant against predators and pathogens. This corroborates recent tests showing the antimicrobial/antiviral activity of polymethylated flavonols (French et al. 1989). Moreover, many similar flavonoids have been shown to possess antibacterial and/or antifungal activities (for examples see Maillard et al. 1987; Mori et al. 1987). Another circumstantial evidence supporting their significance as chemical barrier is provided by the low concentration of flavonoids produced by in vitro

cultures (see Appendix).

5.3. Localization of alkaloids (vindoline)

A number of papers have commonly reported the accumulation of alkaloids in specialized cells (idioblasts) or tissues (laticifers) (e.g. Kutchan et al. 1983, 1986; and for a review, see Wink 1987). The immunodetection of vindoline in idioblasts of Catharanthus was, therefore, anticipated and corroborates with the previous structural observations of Mersey and Cutler (1985), Neumann et al. (1983) and Yoder and Mahlbergh (1976). However, an important accumulation of vindoline was detected in the vacuoles of mesophyll cells as well. Nevertheless, this unexpected result substantiates the ultrastructural study of Eilert et al. (1987) who showed that no conspicious ultrastructural changes were associated with the transfer of cultured cells to media that were defined for alkaloid production. The occurrence of vindoline in non specialized cells suggests that the idioblasts are not required for its storage. In order to evaluate the importance of idioblasts in the storage of vindoline, it will be of interest to develop a technique that can be used to measure its precise subcellular concentration without any leaching of this metabolite.

The results presented here show that vacuoles of differentiated cells of Catharanthus represent the major site of vindoline accumulation. This corroborates with the ability of isolated vacuoles to take up indole alkaloids (Deus-Neumann

and Zenk 1984; 1986; McCaskill et al. 1988; Renaudin and Guern 1982; Renaudin et al. 1985, 1986) and the correlation observed between the differentiation of vacuoles and the appearance of alkaloids (Neumann 1975). The accumulation of vindoline in the vacuole may be attributed to its cytotoxicity. It is generally accepted that cytotoxic compounds may be either compartmented in the cellular vacuole or, in the case of Chrysosplenium, sequestered in the cell wall. Whereas the effect of vindoline on cellular function is yet to be elucidated, its derivatives, vinblastine and vincristine seem to interfere with microtubule organization and inhibit RNA synthesis (Dawson 1986). Thus, the vacuolar accumulation of vindoline may protect the host cells against such detrimental effects.

On the other hand, the specific association of label within the chloroplasts and cytosol suggests recognition by the vindoline antibody of biosynthetic intermediates, some of which have been reported to be cytosolic or chloroplastic (DeLuca and Cutler 1987).

5.4. Cellular, subcellular specialization

In view of biotechnological applications, the low concentration of secondary metabolites produced by cells cultured in vitro is frequently ascribed to the absence of differentiated storage cells and/or specialized tissues. The present investigation has demonstrated that neither the flavonoids of Chrysosplenium nor vindoline of Catharanthus accumulates exclusively in specialized storage cells. However,

this observation does not exclude the general assumption that secondary metabolism is closely linked to the developmental stage of an organism. Differentiation, other than morphological specialization, may be required to confer upon certain cells the capability to synthesize and/or accumulate these secondary metabolites (Böhm 1982)

DeLuca and his collaborators (1985, 1986) have recently demonstrated the developmental regulation of vindoline biosynthesis. Moreover, in Chrysosplenium and Catharanthus, the importance of the process of differentiation is further supported by the fact that regenerated shoots of both plant species produce higher concentrations of secondary metabolites than the undifferentiated cells (see Appendix and Constabel et al. 1982; Endo et al. 1988; Kruegger et al. 1982). The absence of flavonoids in meristematic cell walls of Chrysosplenium (see Fig. 4.13) seems to suggest the requirement of a minimum level of cellular organization for the biosynthesis of these metabolites. Furthermore, cells that accumulate high concentration of either tannins or flavonoids are typically characterized by their rich content of cytoplasmic vesicles in the vicinity of the plasmalemma (see Figs. 4.4, 4.5, 4.11 and 4.12). In comparison with other mesophyll cells, the plasmalemma of these distinctive cells appears more undulated, suggesting intense metabolic activity leading to biochemical differentiation. Due to the poor preservation of Catharanthus membranes, such correlation seems difficult to establish, although a dense labeling was

consistently observed in small vacuoles. From an ultrastructural point of view, these vacuoles may originate from the coalescence of vesicles that may be budded off from the endoplasmic reticulum.

The abundance of cytoplasmic vesicles was also reported to be associated with the development of laticifers (Eilert and Constabel 1985; Eilert et al. 1984, 1986); glandular trichomes (Charriere-Ladreix 1975; 1976; Schneff and Klassova 1972 (cited in Fahn 1979); Wollenweber et al. 1971 (cited in Fahn 1979)) and in cells characterized by their high concentration of anthocyanins (Hrazdina and Wagner 1985; Nozzolillo and Ishikura 1988; Small and Pecket 1982), berberine (Amaan et al. 1986), naphthoquinones (Tabata et al. 1982), phenolic acids (Peterson et al. 1978) and tannins (Baur and Walkinshaw 1974; Stafford 1988). The close association of these secondary metabolites with vesiculation was related to the importance of these membrane structures for the biosynthesis and/or intracellular transport of these metabolites.

Similar functions may be ascribed to vesicles of flavonoid- or vindoline-rich cells. Recent work in our laboratory has suggested that the stepwise O-methylation and O-glucosylation of flavonoids in C. americanum may occur on the surface of a protein aggregate that is loosely attached to a cellular membrane system (see Ibrahim et al. 1987 and refs therein). This hypothesis was based on evidence derived from biosynthetic, enzymic and kinetic studies. The recent

demonstration that the flavonol glucosyltransferase of this tissue seems to be associated with vesicular structures lends further support to that hypothesis (Latchinian et al. 1989). In addition, there is increasing experimental evidence indicating that many enzymes of secondary metabolism are either integrated with or bound to, cellular membranes (Czichi and Kindl 1977; Hrazdina et al. 1980, 1984, 1987; Jonhson et al. 1983; Roos and Luckner 1986) or associated with the plastids (Charriere-Ladreix 1978; Charriere-Ladreix et al. 1981; Cosio and McClure 1984; DeLuca and Cutler 1987; Ranjeva et al. 1977; Wink and Hartmann 1982). However, glucosylated flavonols were rarely detected in vesicles by the use of immunocytochemistry. This result suggests that intermediates, not recognized by the antibody, may be transported to the vicinity of the cell wall by these vesicles. This has been substantiated by biochemical evidence indicating that glucosylation of polymethylated flavonols is a later step in flavonoid biosynthesis. Polymethylated flavonol aglycones, which are more lipophilic, are transported readily into vesicles. A vesicular transport of intermediates would also explain the caffeine-positive reaction of vesicles (see section 4.2.2) despite being less specific reagent than antibodies. Taken together, these several lines of experimentation seem to demonstrate the importance of Chrysosplenium vesicles for the biosynthesis of flavonoids and the channeling of their intermediates. However, definite proof awaits the localization of intermediates and their

biosynthetic enzymes. To date, there is no concensus on the subcellular site of flavonoid biosynthesis. With the recent evidence that a number of phenylalanine ammonia lyase (Bolwell et al. 1985) and chalcone synthase (Koes et al. 1988; Ryder et al. 1987) genes exist in plant cells, it seems possible that the differential distribution of isoenzymes may contribute to the regulation of secondary metabolism. In order to elucidate this point, future work should be directed at the gene and enzyme levels.

For Catharanthus, the biosynthesis of vindoline in the cytosol and chloroplast (DeLuca and Cutler 1987) contrasts with its accumulation in small vacuoles. This situation illustrates the importance of vesicles in the transport of vindoline from the cytoplasm to the vacuole. Such vesicular transport is further supported by the present work. Furthermore, based on previous uptake experiments of acylated flavonols (Matern et al. 1986), it is possible to assume that the acetylation of vindoline in the cytosol may be required to facilitate and regulate its transport across the tonoplast or across the vesicular membranes. Evidence in support of simple diffusion of alkaloids has been presented for nicotine (Kurdjian 1982), tabernanthine and ajmalicine (Renaudin and Guern 1982) and more recently for vindoline (McCaskill et al. 1988). The diffusion process does not exclude the potential importance of a carrier to mediate and regulate the transport of vindoline into membranes (Deus-Neumann and Zenk 1986; McCaskill et al. 1988).

In this study the parietal and vacuolar sequestration of flavonoids and alkaloids, respectively strongly suggests that these metabolites may interfere with some processes of primary metabolism. Consequently, the expression of secondary metabolism should be regulated within the cell. In relation to previous reports on enzyme localization, the present study seems to indicate that the spatial separation between the sites of biosynthesis and accumulation may play an important role in regulating the expression of secondary metabolism. This also suggests the importance of intracellular transport. However, up to now, a complete picture of this regulation can not be outlined. Many questions remain unanswered and await further work for their elucidation. Better knowledge at the genetic and enzymatic levels is required for the understanding of these processes. Furthermore, the development of mutants which lack the ability to synthesize secondary metabolites should provide a new approach to these problems.

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Appendix 1. Tissue culture of Chrysosplenium americanum

Tissue culture

A high level of contamination was noted in the cultures initiated from excised, surface-sterilized tissues of Chrysosplenium even in the presence of antibiotics. This result was not unexpected due to the semi-aquatic nature of the plant and its tiny character. Similar situation was reported with fruit tissues that received different sterilization procedures. In C. americanum, however, treatments such as soaking in the fungicide benomyl, in a 10% solution of sodium hypochlorite and/or in streptomycin, resulted in a rapid senescence of the tissues. The best compromise for the survival of explants, in the absence of contamination, was obtained after soaking the tissues in a 5% solution hypochlorite for 5 to 7 min, followed by one min in 80% ethanol and finally rinsing with sterile water. However this sterilization procedure seems to increase sensitivity of tissues to light thereby causing a rapid loss of chorophyll resulting in a quiescent culture. Nonetheless, as shown in figure 1b, adventituous buds began to form within three weeks following the first inoculation. Although the loss of chlorophyll was delayed in the dark, however, the lack of illumination inhibited the subsequent caulogenesis. Whereas the highest morphogenic potential was observed with shoot tips and to a lesser extent with nodal segments, both excised internodes and roots failed to proliferate or differentiate under any of the experimental conditions used. In general, the

budding response was fairly consistent, particularly for the explants deposited on B5 medium containing 0.5 mg/L NAA and 1 to 5 mg/L zeatin (Table 1). Similar responses were obtained with other auxins (IAA and 2,4-D) and cytokinins (BAP, kinetin), although browning of explant was frequently observed in the presence of BAP and/or 2,4-D. Plantlets with disproportionate root development and profuse root hairs were especially formed from the explants cultivated in the absence of exogenous auxins.

The organogenic potential of Chrysosplenium tissue culture has been maintained with a good rate of multiplication for several subcultures (at least two years) on B5 medium supplemented with 1mg/L each, of NAA and zeatin (maintenance medium). The direct induction of calli was difficult on Chrysosplenium plants even in the presence of high concentration of auxins. Moreover, these calli did not survive further subculture. Therefore, it was considered important to initiate first a sterile shoot culture on B5 medium, supplemented with 0.5 mg/L NAA and 1.0 mg/L zeatin, which was then subcultured every four weeks on the maintenance medium. Only then, that a callus culture could be obtained through selection of the subcultured explants.

After at least two subcultures on the maintenance medium, sterile explants (entire cultured shoot, stem, shoot or root segments) were transferred on nutrient media in which the ratio of auxin/cytokinin was increased (ratio varying from 1 to 5). However, despite caulogenic activity, calli were

Table 1. Effect of NAA and zeatin on the number of buds formed from apical shoots cultured in different nutrient media*.

MEDIA	ZEATIN (mg/L)	NAA (mg/L) 0	0.5	1
MS	0	4.75	5.25	7.30
	1	6.50	9.00	4.00
	5	7.25	9.00	6.50
	10	7.25	8.00	5.75
Heller	0	7.75	9.75	--
	1	11.00	10.25	7.50
	5	9.75	13.50	10.00
	10	7.25	11.50	9.25
B5	0	16.50	17.25	9.00
	1	18.00	21.50	17.50
	5	12.00	15.50	11.50
	10	11.00	10.00	8.00

* Mean of four replicates of three samples each, with standard deviation values < 20%.

occasionally induced on entire explants. The formation of calli seemed to be dependent on the medium composition as well as on the type and concentration of auxin. Under the experimental conditions used, callus was mainly formed on B5 and rarely on MS or Heller's media. In contrast with IAA, either NAA or 2,4-D was efficient in producing callus tissues, but the latter was rather toxic to the cells. The hormonal concentrations which were found optimal for callus induction amounted to 1 to 2.5 mg/L NAA and 0.25 to 0.50 mg/L zeatin. Callogenesis was, however, neither stimulated by cocconut milk nor by yeast extract. On the other hand, it is interesting to note the high frequency of calli formed on root tips as well as the basal end of the stem. Two types of calli were observed: one fluffy withish type and the other, compact brownish callus; neither of which grew in sufficient amount to allow quantitative measurement.

Flavonoid production

HPLC analysis of intact leaf extracts indicated the presence of five partially methylated flavonol glucosides. Quantitative analysis showed a consistent decrease in the amount of individual flavonoid with increasing age of intact leaves. Intact roots, on the other hand, contained compounds A,D and E only either in small or trace amounts (Table 2). Differentiated shoot cultures exhibited a flavonoid pattern similar to that ot the intact leave, albeit in small amounts, which ranged from 7% for compound C, 9-10 % for A,B & E and 20 % for D. Differentiated root and callus cultures contained

Table 2: Flavonoid composition of intact and cultured tissues of C. americanum.

TISSUE	FLAVONOIDS* (nmol/g fresh tissue) A	B	C	D	E
INTACT TISSUES					
First leaf	217 + 22	38 + 5	88 + 12	102 + 2	158 + 16
Second leaf	88 + 4	15 + 1	43 + 5	48 + 6	66 + 10
Third leaf	29 + 6	3 + 1	12 + 2	14 + 5	15 + 4
Root	23 + 3	---	---	3 + 1	4 + 2
CULTURED TISSUES					
Shoot	20 + 17	4 + 5	6 + 6	18 + 16	15 + 14
Root	1.3 + 1	---	---	0.8 + 1	1.3 + 1
Callus					
first generation	0.26 + 0.3	---	---	0.04 + 0.1	0.11 + 0.1
second generation	0.81 + 0.4	---	---	1.10 + 0.6	2.80 + 2.5

*: A) 5,2´,5´-trihydroxy-3,7,4´-trimethoxyflavone-2´-O-glucoside
B) 5,2´-dihydroxy-3,7,4´,5´-tetramethoxyflavone-2´-O-glucoside
C) 5,5´-dihydroxy-3,6,7,4´-tetramethoxyflavone-5´-O-glucoside
D) 5,2´,5´-trihydroxy-3,6,7,4´-tetramethoxyflavone-5´-O-glucoside
E) 5,5´-dihydroxy-3,6,7,2´,4´-pentamethoxyflavone-5´-O-glucoside.

only trace of compounds A, D and E, a profile characteristic of the intact roots (Table 2). The high standard deviations obtained with the analysis of cultured tissues are not unexpected and may reflect the heterogeneity inherent to tissues cultured *in vitro*. However, it is interesting to note that the subcultured callus exhibited increased amounts of flavonoid compounds (Table 2), thus indicating their *de novo* synthesis. This is further supported by the incorporation of label from [14C]cinnamate into both shoot and callus cultures (Fig. 1).

Figure 1. Photograph of an autoradiogram of the chromatographed methanolic extract of shoot/callus tissue administered 2-14C-cinnamate, solvent 1: toluene-ethyl formate-ethanol-water (60:20:19:1), solvent 2: water-n-butanol-acetone-dioxane (70:15:10:5).