EXTRACTION, PURIFICATION AND DETERMINATION OF SOLASODINE IN CULTURES OF *Solanum mauritianum* Scop.

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from May 1990 to November 1993, under the supervision of Professor J. Van Staden. These studies, except where the work of others is acknowledged, are the result of my own investigation.

Francesca Elisabeth Drewes

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ABSTRACT

Solasodine, a steroidal alkaloid, is used by the pharmaceutical industry in certain parts of the world, as a raw material in the synthesis of steroid drugs. The compound is contained in many members of the genus *Solanum*, including *S. mauritianum* Scop., a common weed in South Africa. The levels of solasodine in three culture systems of *S. mauritianum* under various cultural conditions were examined.

A high performance liquid chromatographic (HPLC) method was developed for the detection of solasodine. In order that low-cost, fixed wavelength ultra-violet detectors could be used, which would make the technique more widely applicable, a derivatization step, namely benzoylation, was included in the sample preparation. An extraction and purification protocol was then established, that complemented the HPLC technique and allowed successful detection of solasodine levels in a whole range of different sample types, including callus, suspension cultured cells, roots, stems and leaves.

The three culture systems examined were callus, suspension and hairy root cultures. The callus system was used to establish which cultural parameters affected solasodine content *in vitro* to the greatest extent. A control culture was grown on a MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol and lacking hormones. This culture contained an average of 9.2 μg g^{-1}DW of solasodine. Many factors, including alteration of the carbon : nitrogen ratio and substitution of Gelrite for agar as the

gelling agent, had no significant effect on the solasodine content of the callus or its growth. Greatly increased solasodine productivity of the callus was recorded when glucose was substituted for sucrose, the medium strength was reduced by half, or certain combinations of the hormones benzyladenine and naphthaleneacetic acid were added to the medium. The maximum levels of solasodine recorded in these cultures, on a per gram dry weight basis, equalled those of the vegetative parts of an intact *S. mauritianum* plant, but were approximately three times lower than those of the green berries.

Suspension cultures could not be grown on the same medium as the callus cultures. Substitution of the vitamin complement of MURASHIGE and SKOOG (1962) with the so-called RT vitamin complement of KHANNA and STABA (1968), resulted in successful growth and maintenance of *S. mauritianum* suspension cultures. The auxin, 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) was included in the medium. None of the suspension cultures grown on this medium, or slight modifications thereof, contained any trace of solasodine. This system could therefore not be used for the synthesis of solasodine *in vitro*.

Hairy root cultures were initiated by inoculation of an excised hypocotyl of an *in vitro*-grown seedling of *S. mauritianum* with a 48 hour culture of *Agrobacterium rhizogenes* LBA 9402. Transformation frequency was extremely low. The transformed roots could be excised and grown successfully on a phytohormone-free medium, either in the solid or liquid form. Solasodine was extracted from hairy roots grown in a full-strength liquid MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 3 % sucrose and 0.1 g l^{-1} *myo*-inositol, a

half-strength such medium and a full-strength medium with 3 % glucose substituting for 3 % sucrose. In the latter medium, growth was very poor, whereas in the other two media, growth was very rapid. Both solasodine content (126 μg g^{-1}DW) and root growth were greatest in the full-strength medium supplemented with 3 % sucrose. This level of solasodine was greater than that found in any of the callus cultures or vegetative parts of the plant and approached that of the green berries of *S. mauritianum*. Overall, of the culture types of *S. mauritianum* tested, the hairy root culture system appears to be most favourable for the *in vitro* production of solasodine.

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GENERAL INTRODUCTION

The Solanaceae is a plant family that has attracted the attention of man throughout the ages, for a host of different reasons. The group encompasses many agricultural food crops, one of the most important being the potato (*Solanum tuberosum* L.). This is the major vegetable in much of the world and is second to no plant in yield of calories per acre. Other edible solanaceous species include the tomato (*Lycopersicon esculentum* Mill.), the eggplant (*Solanum melongena* L.) and the chilli (*Capsicum*) (KUC, 1984). In addition, many solanaceous species are grown in gardens as ornamentals. Of all of these, *Petunia* is probably the most important. Its popularity has resulted in the development of a great variety of colours and shapes among the flowers (HEISER, 1969).

Members of the Solanaceae have long been assigned magical and medicinal properties. Perhaps no plant has had more superstitions connected with it than the mandrake (*Mandragora officinarum* L.). The supposed resemblance of the two-branched root to the human figure assumed great significance during the Middle Ages, and it was considered a cure for all human ills. Also, the plant was thought to influence sexual relations, either as an aphrodisiac or a cure for sterility. In addition, mandrake has narcotic properties and may have been used as one of the first anaesthetics. The seeds of *Datura* were used by the Peruvians for the same reason. Another solanaceous species with narcotic properties, that is utilized today, is tobacco (*Nicotiana tabacum* L.). For a plant that provides neither food nor drink and is considered harmful by many, tobacco has achieved remarkable success. Some people have considered it to be one of the New World's greatest gifts, while

others have suggested that it was the Indians' revenge! (HEISER, 1969). Many of the medicinal properties of solanaceous species are now known to be attributable to the glycoalkaloids they contain. In modern times, some of these compounds have become increasingly important in the pharmaceutical industry, as they can be chemically converted to steroid drugs. The most important of these compounds is solasodine, a steroidal alkaloid that is widespread in *Solanum* species, including *Solanum mauritianum* Scop., an abundant weed in South Africa.

CHAPTER 1

LITERATURE REVIEW

1.1. *Solanum mauritianum* Scop. - Taxonomy, distribution and morphology

The genus *Solanum* has 1500 to 2000 species distributed throughout the world and is one of the largest genera of Angiosperms (FROHN and PFÄNDER, 1984). *Solanum* species can be recognized by their relatively small, flattish, star-shaped or circular flowers, with yellow anthers clustered in a central cone around the style (MUNDAY, 1988). *S. mauritianum* Scop. (Syn. *S. auriculatum* Ait.) is one of 30 species of *Solanum* found growing in Natal (ROSS, 1972). The species has a number of common names, including tobacco tree, groot bitterappel, luisboom and bugweed, the latter being its most frequently utilized name in South Africa (WELLS, BALSINHAS, JOFFE, ENGELBRECHT, HARDING and STIRTON, 1986). The plant (Fig. 1) is a perennial shrub or small tree, up to four metres in height, with woody stems and herbaceous leaves. The grey-green leaves, which are densely covered with hairs, are up to 30 cm long and 15 cm wide. The inflorescence is branched and produces many violet flowers. The green berries that form subsequently, turn a dull yellow at maturity (AULD and MEDD, 1987), and can be produced throughout the year. About 20 to 80 berries are formed from each inflorescence and each berry contains up to 150 seeds, of which 98 % are viable (CAMPBELL and VAN STADEN, 1983).

Figure 1. *Solanum mauritianum* Scop.

Some sources record *S. mauritianum* to be a native of Asia (CAMPBELL and VAN STADEN, 1983; MUNDAY, 1988), while others indicate it to have been introduced from South America (AULD and MEDD, 1987). The plant is now a widespread weed in South Africa, Swaziland (WELLS, BALSINHAS, JOFFE, ENGELBRECHT, HARDING and STIRTON, 1986) and Australia (AULD and MEDD, 1987). It is an efficient competitor for space, water, light and nutriment, thus being able to replace preferred, indigenous vegetation. It can occupy terrestrial dry, terrestrial moist and streambank habitats and will survive in winter rainfall (temperate), all-year rainfall (temperate), summer rainfall (temperate), and summer rainfall (sub-tropical) regions (WELLS, BALSINHAS, JOFFE, ENGELBRECHT, HARDING and STIRTON, 1986). Many *Solanum* species are regarded as potentially toxic, and *S. mauritianum* is no exception.

1.2. Glycoalkaloids of *Solanum*

Most *Solanum* species contain glycoalkaloids (OSMAN, 1980). On removal of the sugar residue by hydrolysis, they yield steroidal alkamines, with the 27 carbon skeleton of cholestane (PRELOG and JEGER, 1953; SCHREIBER, 1973). The aglycone skeleton is either of the solanidane or spirosolane type (Fig. 2).

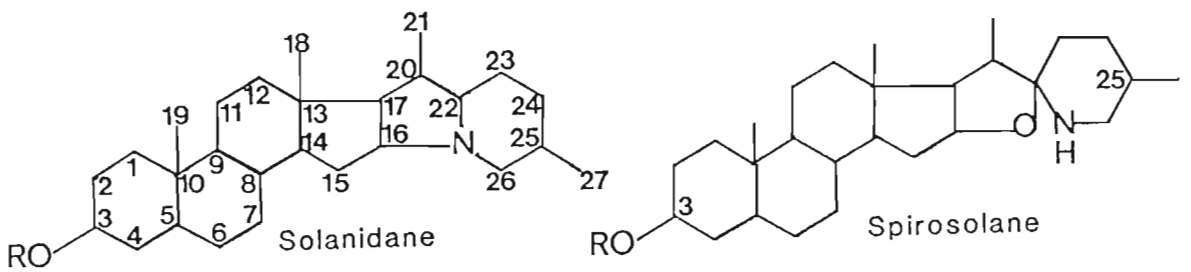

Figure 2. Chemical structures of solanidane and spirosolane

Solasodine (Fig. 3) is of the spirosolane type. Essentially, all glycoalkaloids consist of three entities: A polar, water-soluble oligosaccharide portion of up to five monosaccharide units attached at C3; a non-polar, lipophilic steroidal framework; and a nitrogen heterocyclic moiety that is known to produce various toxic effects (GAFFIELD, KEELER and BAKER, 1991).

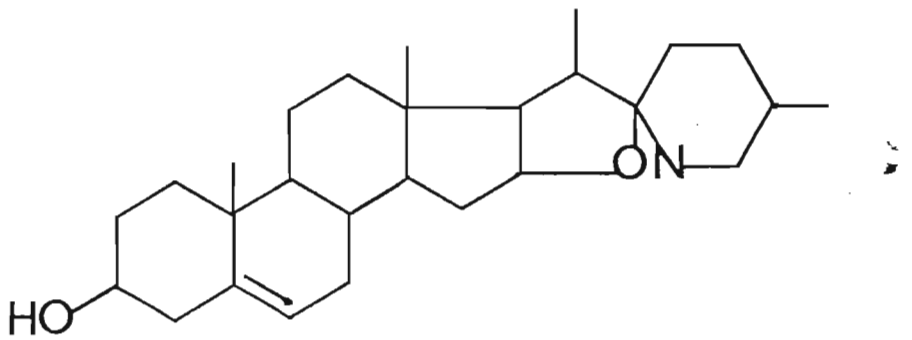

Figure 3. Chemical structure of solasodine

The multiplicity of glycoalkaloids found in *Solanum* species stems from minor modifications of the aglycone structure and various combinations of aglycone and carbohydrate moieties (OSMAN, 1980). Slight alterations in structure or configuration can exert profound effects on their biological properties (GAFFIELD, KEELER and BAKER, 1991). In chemical terms, glucosylation results in enhanced water solubility and reduced chemical activity (HÖSEL, 1981). The two glycoalkaloids most frequently found in solanaceous species are solasonine and solamargine. Solasonine has solatriose as its carbohydrate moiety, which consists of D-galactose, L-rhamnose and D-glucose, while solamargine contains chacotriose, which comprises two L-rhamnose molecules and one D-glucose (OSMAN, 1980).

It was in the 1940's that the characterization of *Solanum* glycoalkaloids was undertaken, with Prelog, Kuhn and Schreiber contributing significantly to our knowledge of glycoalkaloid structures (OSMAN, 1980). Novel steroidal alkaloids are still being isolated from various *Solanum* species. For example:

Solanocardinol from *S. neocardenasii* Hawkes & Hjerting (OSMAN, SINDEN, IRWIN, DEAHL and TINGEY, 1991);

N-hydroxysolasodine from *S. robustum* Wendl. (RIPPERGER and PORZEL, 1992);

25-Isoetioline from *S. canense* Rydb. and *S. fraxinifolium* Dun. (RIPPERGER, 1990);

Capsimine and isocapsicastrine from *S. capsicastrum* Link. (LIN and GAN, 1989).

Solasonine was first isolated from *S. sodomaeum* L. berries by ODDO and COLOMBANO (1905) and solasodine was subsequently obtained by acid hydrolysis. The chemical structure of solasodine was finally fully elucidated by BRIGGS, HARVEY, LOCKER, MCGILLIVRAY and SEELYE (1950).

The Solanaceae encompasses many agricultural crop plants, such as potato, tomato and eggplant. The presence of glycoalkaloids in such foods has always been a matter of some concern and has generated much research in the field. Glycoalkaloids are found in low levels in all tissues of the potato plant, including the tubers (KUC, 1984). They can accumulate to high levels in greened and damaged potatoes (MORRIS and LEE, 1984). Higher levels are found in tubers of

wild species. Thus the use of wild *Solanum* species in potato breeding should be approached with caution, since this may result in potentially hazardous levels of toxic substances (VAN GELDER, VINKE and SCHEFFER, 1988).

1.2.1. Effects of steroidal glycoalkaloids and alkaloids:

The only glycoalkaloids recorded as actually causing human death are those produced by potatoes, namely α-solanine and α-chaconine. The toxicity of potato glycoalkaloids appears to be far greater in man than in other animals studied, although many cases of livestock losses due to ingestion of solanaceous plants have been reported. In man, glycoalkaloid levels of 3 to 6 mg kg^{-1} are said to be lethal. This is equivalent to the toxicity of strychnine (MORRIS and LEE, 1984). Lower doses will result in a variety of symptoms, including headaches, vomiting, severe diarrhoea, exhaustion and abdominal pains. The symptoms may not occur until four to 19 hours after taking the toxic plant material, and in serious cases, nausea and diarrhoea may continue for three to six days. In addition, there are neurological disturbances such as hallucinations, apathy and restlessness, together with convulsions and disturbances of vision (FROHN and PFÄNDER, 1984).

Adverse physiological effects of steroidal glycoalkaloids are thought to stem mainly from membrane disruption, inhibition of acetylcholinesterase, interference with sterol/steroid metabolism, or from combinations of these (RODDICK, 1989). The effects on membranes may be synergistic, for example: Solasonine has little effect alone, but in combination with solamargine, a marked synergism is observed (RODDICK, RIJNENBERG and WEISSENBERG, 1990).

Various adverse effects of compounds extracted from solanaceous species are recorded in Table 1. In general, solasodine is one of the less toxic substances in *Solanum* species.

Table 1. Adverse effects of extracts of *Solanum* species.

COMPOUND	EFFECT	REFERENCE
Solasodine	Teratogen (growth-causing)	VAN GELDER, VINKE & SCHEFFER (1988)
Solasodine glycoalkaloids	Death of hamsters	BAKER, KEELER & GAFFIELD (1989)
Solasodine and solanidane glycosides	Congenital craniofacial malformations in hamsters	KEELER, BAKER & GAFFIELD (1990)
Glycoalkaloids (alkaloids less effect)	Cholinesterase inhibition	BUSHWAY, SAVAGE & FERGUSON (1987)
α-Solanine and α-chaconine	Acetylcholinesterase inhibition	RODDICK (1989)
Solamargine	Disruption of phosphotidylcholine or cholesterol liposomes	RODDICK, RIJNENBERG & WEISSENBERG (1990; 1992)
Solanum melongena L. (eggplant) extract	Hypotensive action on normotensive rats	SHUM & CHIU (1991)

The effects of steroidal alkaloids and glycoalkaloids are, however, not all detrimental. On the contrary, some may be highly beneficial to man, for example: the anti-fungal activity of the alkaloid solanocongestidine against *Candida albicans, Trichophyton rubrum* and *Cryptococcus neoformans* (KUSANO, TAKAHASHI, SUGIYAMA and NOZOE, 1987). Several other positive effects are summarized in Table 2.

Table 2. Positive effects of *Solanum* extracts.

COMPOUND	EFFECT	REFERENCE
Mixture of solasonine, solamargine and solasodine	Antineoplastic activity against Sarcoma 180 in mice	CHAM, GILLIVER & WILSON (1987)
Capsicastrine	Activity against liver damage induced by CCl_4 in mice	LIN & GAN (1989)
Commersonine	Resistance to Colorado potato beetle	SINDEN, SANFORD & OSMAN (1980)
α-Chaconine, α-tomatine and α-solasonine, but not the aglycones	Inhibition of herpes simplex virus Type 1 in tissue culture	THORNE, CLARKE & SKUCE (1985)
Alkaloid fraction of *S. melongena* L. leaves	Analgesic effect in mice (inhibits acid-induced writhing)	VOHORA, KUMAR & KHAN (1984)
S. panduraeforme E. Mey (used in folk medicine)	Helps cure toothache, rheumatism, stomach ailments	DÖPKE, MATOS & DUDAY (1987)
S. lyratum Thunb. plants	Remedy for various cancers in the Shanghai region of China	YAHARA, MOROOKA, IKEDA, YAMASAKI & NOHARA (1986)
β-Lycotrioside and β-lycotetraoside	Inhibit growth of a human cervical cancer cell line	MURAKAMI, EZIMA, TAKAISHI, TAKEDA, FUJITA, SATO, NAGAYAMA & NOHARA (1985)

The most important advantageous property of the steroidal alkaloid solasodine, lies not in any positive effect the compound itself has, but rather that it can be used as a precursor for the synthesis of medicinal steroids.

1.2.2. Use of plant raw materials as steroid precursors:

Medicinal steroids are manufactured by American and European companies and by government agencies in Russia and China. The American and European companies have traditionally used the steroid diosgenin as their principal starting compound, which they obtain from the Mexican yam, a *Dioscorea* species (BRADLEY, COLLINS, CRABBE, EASTWOOD, IRVINE, SWAN and SYMON, 1978). The diosgenin is isolated from the plant material and then chemically converted to the desired product. This is generally cheaper and simpler than total synthesis of steroids. The need for diosgenin arose in the 1940's, when no commercial source of raw material existed, and it has remained a favoured synthesis intermediate in the pharmaceutical industry for over 40 years. During this time, large quantities of *Dioscorea* rhizomes were collected from the wild in eastern Mexico and this country became the world capital of the steroid intermediates industry. Attempts were made to cultivate the yams, but allowances had to be made for the support of the long woody vines (up to 30 m) and for removal of the rhizomes from soil depths up to one metre at harvest. As the distances between the jungle and the yam processing centres increased, so the cost of diosgenin increased. Also, Mexico attempted to protect its national industry and so supplies of diosgenin to Europe and America dwindled (DAWSON, 1991). These factors, together with an unwillingness to rely on a single supplier and the desire to use internal production to conserve foreign exchange, led to a search for alternative raw materials, one of which is solasodine (MANN, 1978).

1.2.3. Distribution and levels of solasodine in plants:

The potential use of solasodine as a raw material for the synthesis of steroid drugs has led to the large scale screening of solanaceous species. In 1980, WEILER, KRÜGER and ZENK conducted an extensive survey of herbarium leaf material of *Solanum* species, using a sensitive and specific radioimmunoassay. They found about 130 of the species tested to yield between 0 and 0.1 % solasodine and the remaining 82, from 0.11 to 4.4 % solasodine. The main candidates that have been considered for the commercial production of solasodine are *Solanum aviculare* G. Forster, *S. khasianum* C.B.Clarke, *S. laciniatum* Ait. (MANN, 1978) and perhaps *S. marginatum* L.f. (BRADLEY, COLLINS, CRABBE, EASTWOOD, IRVINE, SWAN and SYMON, 1978). *S. mauritianum* (bugweed) is reported to contain 0.5 % solasodine in the leaves and 1.7 % in the green fruits (SCHREIBER, 1963 in MANN, 1978 survey). Although this is lower than that of the *Solanum* species currently being exploited, it is considerably greater than average, making *S. mauritianum* a species definitely worth investigating.

The distribution of solasodine glycosides within the plant varies. In *S. aviculare* and *S. laciniatum*, the younger leaves and fruit yield most of the solasodine (LANCASTER and MANN, 1975), whereas in *S. khasianum*, the leaves only yield traces of solasodine and it is the fruits that are collected for solasodine extraction (MANN, 1978). The solasodine glycoside content also varies during the development of the plant. For example, in *S. laciniatum*, all parts of the plant yielded low concentrations of solasodine 10 weeks after germination, but thereafter high concentrations developed in the leaves (mature and immature). Roots and

stems yielded much less alkaloid. The highest solasodine concentrations were recorded in the immature berries (LANCASTER and MANN, 1975). In most *Solanum* species, the green fruits are the organs that yield the highest solasodine levels. However, there are exceptions, for example: In *S. sysimbrifolium* Lamk., the mature, scarlet berries give the greatest yield (PANDEYA, SARATBABU and BHATT, 1981a). The localization of solasodine within the actual fruit has also been examined in *S. aculeatissimum* (Jacq.). It was found that the fruit pericarp contained high levels of free solasodine, whereas its glycosides were most concentrated in the seeds themselves (KADKADE, RECINÓS and MADRID, 1979). This is one of the few species that contains solasodine in its free form.

1.2.4. Utilization and manipulation of solasodine in Solanum species:

Solanum plants are cultivated for their glycoalkaloid content in many different parts of the world, especially Hungary, India, New Zealand and Australia. One problem that has frequently limited the cultivation of certain species is the presence of vicious spines, which greatly hamper the harvesting of the plants. This includes *S. aculeatissimum* (Jacq.) (KADKADE, RECINÓS and MADRID, 1979), *S. sysimbrifolium* Lamk. (PANDEYA, SARATBABU and BHATT, 1981a) and the high-yielding species *S. khasianum* C.B.Clarke (MANN, 1978). *S. mauritianum* lacks such spines. Another problem is that solasodine yield and plant growth may differ in different regions, due to the influence of different environmental conditions. For example, in India, it was found that solasodine content was higher in berries of *S. khasianum* collected from the plains and lower altitudes, than in those at high altitudes (CHAUDHURI and CHATTERJEE, 1979). Shorter

mevalonate, isopentenyl pyrophosphate, farnesyl pyrophosphate, squalene and cholesterol (SHARMA and SALUNKHE, 1989). The subsequent biochemical steps have not been established (WALLER and DERMER, 1981). It has been suggested that solasodine is synthesized from cholesterol, possibly through the intermediate formation of diosgenin (VICKERY and VICKERY, 1981). However, the mechanism by which the nitrogen is introduced into solasodine remains unknown (PETTERSON, HARRIS and ALLEN, 1991). Phosphorylation of mevalonic acid is the primary point at which control of terpenoid and steroidal alkaloid biosynthesis operates (VICKERY and VICKERY, 1981).

1.3.3. The importance of secondary metabolites:

The separation of the processes generating plant compounds into primary and secondary metabolism is primarily historical, and in the light of present day knowledge, this distinction is rather arbitrary. The classic definition of a secondary product is that it is a substance that appears to have no explicit role in the internal economy of the organism that produces it (WILLIAMS, STONE, HAUCK and RAHMAN, 1989). However, many so-called secondary products are now thought to be very necessary to plant life. Because of the overwhelming variety of secondary metabolites, there is not necessarily any single function for these compounds, or even functions for all of them. Various possible reasons have been put forward concerning the raison d'être of secondary metabolites:

i) They may have arisen as a result of a mutation and be neither beneficial nor harmful to the organism. Such mutations, however, are not an efficient

route for increasing the fitness of an organism to survive. Since a highly ordered and complex system is necessary to produce such a compound, this possibility goes against all theories of natural selection (WILLIAMS, STONE, HAUCK and RAHMAN, 1989).

ii) Secondary products may be the manifestation of evolution in progress (HASLAM, 1986). Thus, they may in future be modified and become important to the producers' survival. Again, this hypothesis has little support, since it is regarded as contrary to natural selection that such highly-programmed structures could have arisen to serve a future role (WILLIAMS, STONE, HAUCK and RAHMAN, 1989).

iii) Secondary products could be waste or detoxification products (HASLAM, 1985; 1986). Indeed, most plants cannot degrade certain aromatic amino acids and hence store large amounts of secondary products derived from compounds such as L-tryptophan, L-tyrosine and L-phenylalanine (LUCKNER, 1984). WILLIAMS, STONE, HAUCK and RAHMAN (1989) argue against this theory, in view of the tremendous diversity and complexity of these compounds, and the complex processes necessary to produce them. There appears to be more evidence to support this theory in animals than in plants (LUCKNER, 1984).

iv) Secondary metabolism may maintain basic metabolism in circumstances when the products of primary metabolism cannot, through nutritional imbalances, be used for replication ie: act as an overflow (BU'LOCK, 1980). Thus, the

process of secondary biosynthesis is advantageous, rather than its products, although BU'LOCK (1980) did not exclude the possibility that the products themselves may have a use in the plant.

v) The secondary metabolites may have had a functional role in the organism in the past. This hypothesis is impossible to test and has generally been rejected (WILLIAMS, STONE, HAUCK and RAHMAN, 1989).

vi) Secondary metabolites may have evolved in order to provide ecological advantage to the organisms that produce them. This theory is the hypothesis that has received much favour and has led to the discovery of such functions for many secondary metabolites, but by no means all.

Animals are mobile and depend on this to obtain food and to evade predators. Since plants lack mobility, they must evolve alternative strategies for survival, and it is thought that this is where certain secondary metabolites play a role in plants (BELL, 1981). These compounds may:

i) Act as hormones, regulating plant growth (VICKERY and VICKERY, 1981). They may, however, be of significance in one organism, but not in another, or have deviating usage in other organisms (LUCKNER, 1984).

ii) Function as wounding substances. For example, traumatic acid is responsible for the healing of damaged tissue.

iii) Attract insect and bird pollinators or seed dispersal agents, due to the colours of flavonoids and carotenoids (VICKERY and VICKERY, 1981), and also the presence of nectar and scents (LUCKNER, 1984).

iv) Act as feeding deterrents (MANN, 1987), by conferring on the plant a bitter, astringent or aromatic taste, which repels herbivores (LUCKNER, 1984). As mentioned previously (Section 1.2.1), the presence of the glycoalkaloids commersonine and dihydrocommersonine in *Solanum chacoense* Bitter leaves, increases resistance of the plant to the Colorado potato beetle (SINDEN, SANFORD and OSMAN, 1980). Many secondary products can actually adversely affect the fitness of the herbivore, if the plant is ingested, for example cardiac glycosides and alkaloids (LUCKNER, 1984).

v) Confer competitive advantage of the plant, by chemically preventing growth of other plants in the immediate vicinity ie: act as allelopaths (LUCKNER, 1984). Often, self-toxicity is evident, but the associated benefits usually outweigh the disadvantages, especially for species with a short life span (MANN, 1987).

vi) Protect the plant from microbial attack, by being synthesized in the infected tissue or its immediate neighbourhood, thus warding off the attack, since they inhibit growth of the pathogen. These compounds are known as phytoalexins (LUCKNER, 1984).

Thus, it has become increasingly clear, that many secondary products are of major importance in the life of the plant, especially with respect to its interactions with other organisms in the environment. However, it is possible, and seems likely, that some secondary metabolites are of no value to the plant and may never be assigned any function. Whether or not secondary metabolites have a function on plants, many may still have a use to man, as commercially valuable chemicals. These chemicals may be extracted from the plants directly, or be produced under controlled condition in plant tissue cultures. It is this latter aspect that has been addressed in the current study.

1.4. Production of secondary products in culture

The plant products used by man do not only include medicinal agents, but also plant pigments and perfumes, that are very important in the food and cosmetic industries (STAFFORD, 1991). Folk medicine, effective screening and an element of good fortune are regarded as necessary in discovering new natural products that are of importance. The idea that plant tissue cultures could be used as a source of these specific and often complex chemicals, has intrigued researchers since the mid 1950's (DOUGALL, 1986).

There are several advantages that the production of secondary products in a controlled, tissue culture system has over the extraction of these chemicals from plants:

i) Biomass production is more rapid than for whole plants (HEINSTEIN, 1986).

ii) Product quality is more uniform (CURTIN, 1983), since nutritional and environmental conditions can be controlled more easily (HEINSTEIN, 1986).

iii) Year-round production of the product ensures continuity of supply (RHODES, ROBINS, HAMILL and PARR, 1986).

iv) Oscillations in availability of chemicals due to the required long periods (one to five years) of plant growth before harvesting of certain plants, are eliminated (DOUGALL, 1986).

v) Disease and drought do not pose problems (BRAMBLE, GRAVES and BRODELIUS, 1990).

vi) Uncertainties concerning price and availability due to social and political changes are eliminated (HEINSTEIN, 1986).

vii) In those cases where the plant is rare or difficult to grow, the supply could be increased to meet the demand (CURTIN, 1983).

viii) Cultures are usually less complex in organization than entire plants and so the product is recovered more easily (ANDERSON, PHILLIPSON and ROBERTS, 1986).

ix) Novel compounds with potentially more desirable activities may be produced

(HEINSTEIN, 1986).

It therefore appears as if production of chemicals in tissue culture systems could be recommended in instances where chemical synthesis of the desired product is not feasible. The suitability of tissue culture systems for secondary production, however, depends on whether or not they can be efficient and cost-effective. Plant cell culture is very labour intensive and the cost of maintaining sterility is high (ROBERTS, 1988). Therefore, the compound that is required should preferably be one that is expensive to obtain by any other means (SCRAGG, 1986). It has been suggested (CURTIN, 1983), that only products valued at more than $1000 per kilogram are worth producing by these means and that the volume required should preferably be small. Diosgenin has been included on lists of products that could realistically be produced in culture (SCRAGG, 1986).

One of the most important factors in the economics of any process, is productivity. It is this that has been the stumbling block in the development of many tissue culture systems, since levels of chemicals produced in culture are generally lower than those of the intact plant. The number of plants in which this has been recorded, is too numerous to mention. The important fact, however, is that in some instances, levels equal to or greater than those in the parent plant have been recorded (Table 4).

With the improved understanding of cultural requirements and the introduction of cell line selection, the number of plants in which the levels of secondary metabolites approach or exceed those in the whole plant, has steadily increased (ANDERSON,

PHILLIPSON and ROBERTS, 1986).

Table 4. Yields of secondary products from certain cultures and whole plants (adapted from ANDERSON, PHILLIPSON and ROBERTS, 1986; and STAFFORD, 1991).

Chemical	Plant	Yield (% DW)	
		Culture	Plant
Anthraquinones	*Cassia tora* L.	6	0.6
Glutathione	*Nicotiana tabacum* L.	1.0	0.1
Ajmalicine	*Catharanthus roseus* (L.) G.Don.	1.0	0.3
Serpentine	*Catharanthus roseus*	0.8	0.5
Rosmarinic acid	*Coleus blumei* Benth.	15	3
Ginsenosides	*Panax ginseng* C.A.Meyer	27	4.5
Shikonin	*Lithospermum erythrorhizon* Sieb. et Zucc.	12	1.5
Diosgenin	*Dioscorea deltoidea* Wall.	2	2
Berberine	*Coptis*	11.4	10
Caffeine	*Coffea*	1.6	1.6
Pseudoephedrine	*Ephedra*	2.25	0.60

The encouraging and oft-quoted success story is that of the commercial production of shikonin in a cell culture system: Shikonin is a traditional medicine in Japan, where it is used for its anti-bacterial and anti-inflammatory effects, primarily for the

treatment of burns and haemorrhoids. The compound is bright red and is thus also used as a dye in the cosmetic and pharmaceutical industries. Conventional shikonin production is problematic, since the plants can only be harvested after five to seven years. Therefore, the price for shikonin was very high and so Mitsui Petrochemical Industries deemed it worthwhile to consider tissue culture production methods. After comprehensive trials, Yasuhira Fujita and his co-workers managed to increase productivity 13-fold (CURTIN, 1983). In 1983, Mitsui Petrochemical Industries commenced with the commercial production of shikonin by cells of *Lithospermum erythrorhizon* Sieb. et Zucc.

An additional encouragement to researchers in this field, is that from 1980 - 1984, 39 Japanese patents were taken out on useful secondary metabolites (FUJITA and TABATA, 1987). It was therefore deemed worthwhile to attempt to produce solasodine in a tissue culture system, in the present study.

The approach used for secondary product synthesis in culture is generally rather empirical. This is of necessity, since very little is known about the control of secondary product synthesis, at a biochemical level. Various factors have been identified as affecting secondary product synthesis *in vitro*, and the effect of some on solasodine synthesis will be examined in the present study. The major factor is the composition of the medium (CONSTABEL, 1988). This includes:

i) Carbon source - Many different carbon sources are able to support growth of cell cultures, but the highest growth rates are usually obtained with sucrose and glucose (KURZ and CONSTABEL, 1985; KURZ, 1986; FOWLER, 1986).

Frequently, although both compounds support similar growth rates, sucrose leads to a higher level of secondary metabolite synthesis than glucose. The mechanism of this differential effect remains puzzling, since sucrose is hydrolyzed to glucose and fructose before entry into cultured cells (FOWLER, 1986). It is not only the nature of the carbohydrate source, but also its concentration that affects secondary product synthesis. Typically, an increase in production has been observed as sucrose concentration increases from two to ten percent (COLLIN, 1987). If carbohydrate levels are too low, the available substrate may be used entirely for cell growth and not for secondary product synthesis (KURZ, 1986).

ii) Nitrogen source - Nitrate and ammonia are the main sources of nitrogen that are used in tissue culture media, although organic sources such as urea, amino acids and casein hydrolysate are also employed (KURZ and CONSTABEL, 1985; KURZ, 1986). The level and nature of the nitrogen source have been observed to have marked effects on growth, cell division and product synthesis. There is little consistency in the results of changing levels of the nitrogen source, since changes are often reported to have different effects on closely-related products in a single plant species (FOWLER, 1986). When manipulating microbial systems for product synthesis, the carbon:nitrogen ratio has been found to be important, and this has been extended to plant systems. Even slight alterations in the carbon:nitrogen ratio can have an influence on secondary metabolite synthesis, especially if the change favours resumption of growth of cells that have entered the stationary phase (KURZ, 1986).

iii) Phosphorus - Excessively high concentrations of phosphate have been found to inhibit secondary metabolite synthesis in plant cell cultures (KURZ and CONSTABEL, 1985). Thus, limitation of phosphate has frequently been used to increase product yield (COLLINGE, 1986). It is thought that it is only when intracellular phosphate levels are below inhibitory concentrations, that enzymes such as tryptophan decarboxylase, which are responsible for linking primary to secondary metabolism, will be activated and product formation commence (KURZ, 1986). Unfortunately, data on the effects of phosphate limitation do not all point in the same direction (FOWLER, 1986).

iv) Macro- and micronutrients - Secondary metabolism can also be affected by alterations in the concentration of macro- and micronutrients in the medium (COLLINGE, 1986), but little detail is available on the subject.

v) Plant growth regulators - It has been said (FOWLER, 1985) that the influence of growth regulators on secondary product synthesis *in vitro*, has been studied more, but is the least understood, of all the areas of medium composition. Subtle changes, both qualitative and quantitative, may have profound effects on the nature and pattern of product synthesis (FOWLER, 1986). Plant growth regulators do not react with intermediates of biosynthetic pathways, but appear to be able to shift conditions in favour of product formation. The synthesis of several secondary metabolites can be affected by the same hormone (KURZ and CONSTABEL, 1985). Since the molecular basis of action of plant growth regulators is still not fully elucidated, it is difficult to judge their role in the synthesis of secondary

metabolites. The classes of plant growth regulators most commonly added to tissue culture media are the auxins and cytokinins, either in their natural or synthetic forms. Frequently, a reduction in auxin concentration or switch to a weaker auxin, results in a reduction in growth rate and an increase in secondary metabolite production (COLLINGE, 1986; BECKER, 1987). The effect of cytokinins on secondary metabolite synthesis is variable (COLLINGE, 1986).

In addition to the composition of the culture medium, external factors such as light and temperature are known to influence synthesis of secondary metabolites *in vitro*. A decrease in temperature to between five and 15°C, is often reported to result in an increase in secondary production. It is thought that this may be due to inhibition of growth under these conditions (COLLIN, 1987). The effect of light on both growth and secondary metabolite production in plant cultures has frequently been studied. However, it is difficult to draw any conclusions, since light sources, quality and quantity vary tremendously (KURZ and CONSTABEL, 1985; KURZ, 1986). Another factor that influences secondary metabolite synthesis, is stress. This may be induced by so-called abiotic elicitors, such as ultraviolet irradiation, osmotic shock or heavy metal ions (CONSTABEL, 1988). Alternatively, biotic elicitors may be added to the medium. These are primarily extracts of fungi and fungal cell wall material (FOWLER, 1986). They trigger the production of phytoalexins, which contribute to the plants' defence against disease. In tissue culture systems, these elicitors have been used to stimulate the production of known phytoalexins and of metabolites which have no known, or proven, role in defence. There are limits to the use of fungal elicitors, since they appear to be able to trigger

the synthesis of only certain metabolites in plants (COLLINGE, 1986).

All the afore-mentioned factors interact with one another and influence the final level of the secondary metabolite of interest, in the tissue culture system used. This empirical approach has been successful in increasing levels of secondary products *in vitro*, beyond those initially observed, and is the approach that will be followed in the current study. As our knowledge of the pathways and enzymes involved in the biosynthesis of secondary metabolites increases, so slightly less empirical approaches, such as the addition of precursors, induction or stimulation of enzyme activities and the inhibition of certain metabolic pathways (RIDEAU, 1987), will no doubt become more applicable. With the lack of knowledge of the details of solasodine synthesis at the present time, however, use of such techniques is not feasible in the present study.

1.5. Direction of research

Solasodine appears to be a realistic alternative to diosgenin in the steroid drug synthesis industry. The ready availability of *Solanum mauritianum*, and the reported presence of above-average levels of solasodine in its green berries, suggest that this plant is worth considering as a potential source of solasodine. Three possible approaches are: Firstly, to harvest the green berries from areas where the plant is growing as a weed, which would have the added advantage of acting as a weed control strategy; Secondly, to cultivate the plant and harvest the berries from the fields; and thirdly, to produce the product intensively under controlled conditions, for example in tissue culture, which would prevent the possibility of

bugweed escaping from cultivation and becoming even more of a pest than it already is. The first and second approaches are presently being investigated elsewhere in South Africa. It is the third approach that forms the basis of this study. However, prior to examining the synthesis of solasodine *in vitro*, a suitable means of detection of solasodine was required, since many of the published techniques present the researcher with only moderately sophisticated equipment, with difficulties (Chapter 2).

With these considerations in mind, the objectives of the study were to:

i) Develop a high performance liquid chromatographic (HPLC) technique for the determination of solasodine.

ii) Establish extraction and purification protocols to complement the HPLC method.

iii) Determine levels of solasodine in a variety of culture types and under a range of cultural conditions.

CHAPTER 2

BENZOYLATION AND HPLC DETERMINATION OF SOLASODINE

2.1. Introduction

Since the increase in interest in solasodine due to its potential as a steroid precursor, many different techniques have been used for the detection of solasodine. Some have been qualitative only, and others quantitative, depending on the purpose for which the technique was required. Thin layer chromatography (TLC) is a simple, yet very effective technique, which has been widely used to monitor the presence or absence of solasodine (VÁGÚJFALVI, MARÓTI and TÉTÉNYI, 1971; KOKATE and RADWAN, 1979; UDDIN and CHATURVEDI, 1979; PANDEYA, SARATBABU and BHATT, 1981b) or as a preparative step (KADKADE, RECINÓS and MADRID, 1979; KOKATE and RADWAN, 1979; JAIN and SAHOO, 1981a). Spectrophotometric techniques are frequently used to obtain a rapid estimate of solasodine levels in plant tissue (RODDICK and MELCHERS, 1985). The colour reactions most commonly used are those involving methyl orange, using the protocol developed by BIRNER (1969), (HOSODA, ITO and YATAZAWA, 1979; CRABBE and FRYER, 1982; BHATT, BHATT and SUSSEX, 1983) or bromothymol blue (LANCASTER and MANN, 1975; NIGRA, CASO and GIULIETTI, 1987).

More sophisticated techniques that have been developed for solasodine analysis include radioimmunoassays, which are so sensitive that as little as 0.5 mg of

material is sufficient for a quantitative analysis (WEILER, KRÜGER and ZENK, 1980), gas chromatography (GC) using packed (CARLE and REINHARD, 1980; HOSODA and YATAZAWA, 1979) or capillary columns (LAWSON, ERB and MILLER, 1992), and high performance liquid chromatography (HPLC). These techniques are distinct from those such as melting point determinations, infra-red, ^{1}H-Nuclear magnetic resonance (NMR; DÖPKE, MATOS and DUDAY, 1987), ^{13}C-NMR (ZEIGAN, VOIGT, HILLER and CUONG, 1987; RIDOUT, PRICE, COXON and FENWICK, 1989) spectroscopy and mass spectrometry (DÖPKE, MOLA, MORAN, PALOMINO, BASTERECHEA and COLL, 1984), which are used for identification of solasodine in single samples, rather than as routine analytical techniques.

The high molecular weight and thermal instability of alkaloids and glycoalkaloids present a problem with respect to their analysis by GC (COXON, 1984), but not HPLC. FRIEDMAN and DAO (1990) indicate that HPLC is increasingly being used to analyze both individual glycoalkaloids and their hydrolysis products in potato plants and commercial potato products. This technique was the one chosen for the detection of solasodine in the present study.

The main obstacle to the HPLC determination of solasodine and glycoalkaloids is that they absorb at the low wavelength end of the ultraviolet (UV) spectrum (CRABBE and FRYER, 1980). This means that the UV detector attached to the HPLC must operate efficiently at less than 210 nm, which is not possible with many of the low-cost, single wavelength, UV detectors available. Such fixed wavelength detectors use the intense, 254 nm radiation from relatively inexpensive mercury arc

lamps (JOHNSON and STEVENSON, 1978). At the lower wavelengths, when using a high quality UV detector, the possible choice of solvents is limited to mixtures of water, acetonitrile, tetrahydrofuran, low molecular weight alcohols and saturated hydrocarbons. Even with these solvents, only isocratic elution is possible if stable baselines are desired (COXON, 1984). If gradient elution is attempted, the baseline change is so large compared with peak size, that peak shape and integration are detrimentally affected (CRABBE and FRYER, 1980). Numerous HPLC studies have been conducted with a view to determining glycoalkaloid levels in potatoes (BUSHWAY, BUREAU and KING, 1986; CARMAN, KUAN, WARE, FRANCIS and KIRSCHENHEUTER, 1986; JONKER, KOOPS and HOOGENDOORN, 1992). However, analysis of aglycones has met with less success. FRIEDMAN and LEVIN (1992) attempted to develop a single assay for both glycoalkaloids and aglycones, but found that column and eluent requirements prevented them from realizing their objective. Despite the limitations, a few HPLC techniques have been developed for solasodine, but all use UV detectors operating at wavelengths less than 210 nm (CRABBE and FRYER, 1980; CHAM and WILSON, 1987; HERBRETEAU-LEMONNIER, ROSSIGNOL, ROSSIGNOL, SIHACHAKR and HAICOUR, 1989). As an alternative, EMKE and EILERT (1986) used a refractive index detector.

It was decided to use a different approach, namely one involving the derivatization of solasodine, such that the product would absorb at a higher UV wavelength. While this would increase the time and cost of analysis, it would enable determination of solasodine with a wider range of HPLC systems. This principle has been applied in the analysis of digitalis glycosides, where 4-nitrobenzoyl

chloride was used as reagent (NACHTMANN, SPITZY and FREI, 1976a; b) and in the analysis of steroids in urine and blood samples, where benzoyl chloride was used (FITZPATRICK and SIGGIA, 1973). On recommendation of Prof. S.E Drewes of the Chemistry Department, University of Natal, Pietermaritzburg, derivatization using benzoyl chloride was attempted in the current study. The final derivatization and HPLC procedures have been published (DREWES, VAN STADEN and DREWES, 1992).

2.2. Materials and Methods

The pure solasodine used in the development of the HPLC technique was obtained from Sigma, as was the benzoyl chloride. All the solvents used in the HPLC studies were HPLC grade.

A Varian Model 5000 liquid chromatograph coupled to a UV50 variable wavelength detector and a Vista 401 Chromatography Data System were used for the HPLC determinations. Throughout, the column used was a Hypersil 5 ODS (25 cm x 4.6 mm i.d.) which is a C-18 reverse phase column with a relatively low polarity. The details of the methods used will be described concurrently with the development of the technique.

2.3. Method Development and Discussion

2.3.1. Initial benzoylation and HPLC determination of solasodine:

Initially, it was attempted to use techniques that had been applied successfully in the benzoylation of polyamines (FLORES and GALSTON, 1982) and animal steroids (FITZPATRICK and SIGGIA, 1973), or nitrobenzoylation of digitalis-glycosides (NACHTMANN, SPITZY and FREI, 1976a; b) for solasodine. This was not successful, due to the different chemical properties of the compounds. The chemical properties of solasodine, such as solubility, were therefore examined and a technique developed specifically for this compound.

By benzoylating solasodine, the aim was to attach a benzoyl moiety to the compound, thus providing a suitable chromophore to allow detection of the complex at a wavelength within the common operating range of most UV detectors.

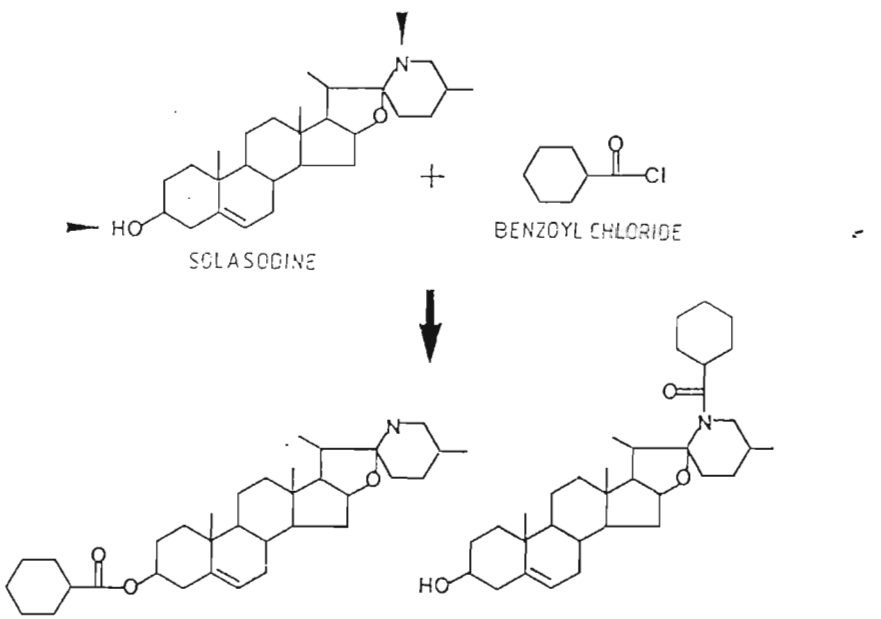

Figure 7. Positions at which solasodine could be substituted with a benzoyl moiety from benzoyl chloride.

The reaction could potentially occur at two positions on the solasodine molecule (Fig. 7), namely at the hydroxyl group via an esterification reaction, or at the nitrogen atom. It was predicted, therefore, that two, or even three peaks might be obtained on an HPLC trace, representing solasodine monosubstituted at either position, or a disubstituted product. A byproduct of the reaction would be hydrochloric acid.

In initial tests to determine the solubility of solasodine in a range of solvents, it was found to be insoluble in water, slightly soluble in alcohols and readily soluble in organic solvents such as ethyl acetate. In the first trial, therefore, 5 mg of pure solasodine was dissolved in 1 ml of ethyl acetate in a tapered glass tube and 8 μl benzoyl chloride added. Since hydrochloric acid would be produced if the reaction proceeded, it was necessary to add a base, in order to neutralize the reaction mixture. Due to the insolubility of solasodine in water, an organic base, triethylamine, was chosen. The reaction mixture was warmed for five to ten seconds in a 50°C waterbath, then left to react at room temperature for 30 minutes. A control was run concurrently, containing all the reagents and solvents, but no solasodine. To terminate the reaction, distilled water was added, which would react with any remaining benzoyl chloride to form water-soluble benzoic acid. The aqueous (lower) layer was then removed by pipetting and another 1 ml of water added and removed, to wash away any remaining reagents. The ethyl acetate fraction was dried over anhydrous sodium sulphate, decanted and concentrated to dryness under nitrogen. Finally, the sample was redissolved in 500 μl HPLC-grade methanol, in which the putatively benzoylated product appeared to be readily soluble, in preparation for HPLC analysis. The control was processed in exactly

the same way.

Since solasodine is a relatively non-polar molecule, with poor solubility in aqueous or alcoholic solutions, 100 % acetonitrile was initially used as the solvent, at a flow rate of 1 ml min^{-1}. Detection was at 254 nm and with 0.05 absorbance units full scale (AUFS), at which sensitivity an even, stable baseline was obtained. A 20 μl aliquot of the control sample was then injected and no peaks were obtained, other than those eluting with the void volume. However, when 20 μl of the sample were injected, two distinct peaks eluted (Fig. 8). They were arbitrarily assigned the names A and B.

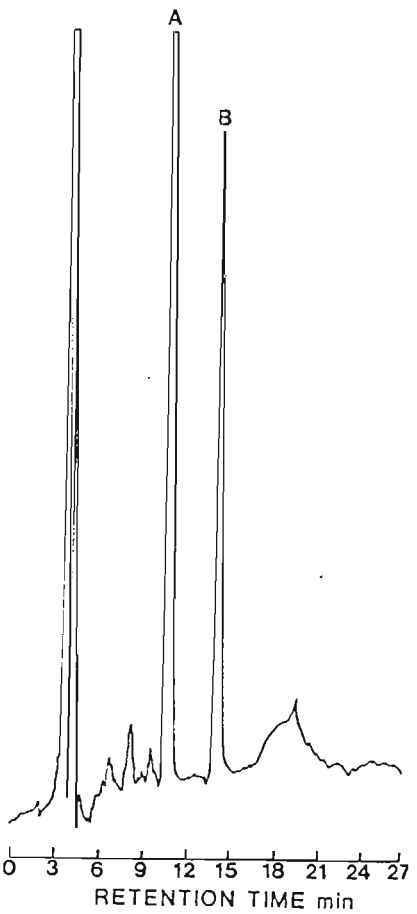

Figure 8. HPLC trace obtained when elution of benzoylated solasodine was first attempted, with 100% acetonitrile.

The remainder of the sample was then injected into the HPLC in successive injections. Following each injection, the fractions corresponding to the retention times of the two peaks (A and B) were collected manually in glass tubes. All fractions A and all fractions B were collected in the same tubes and then concentrated to dryness under nitrogen. These tubes were sent to the Processing and Chemical Manufacturing Technology Division of the CSIR, where the samples were subjected to low resolution mass spectrometry and accurate mass measurement, using a Varian MAT 212 mass spectrometer. The accurate mass measurement of peak A was found to be 621.3832 and of peak B, 517.3546. Since solasodine has a molecular weight of 413.7 and the benzoyl moiety, 104.5, peak A corresponds to disubstituted solasodine and peak B to the monosubstituted derivative. Since both possible monosubstituted derivatives would have the same mass, it was not possible to determine whether peak B was a mixture of the two forms, or whether one was dominant. However, in subsequent trials, peak A was consistently found to be larger than peak B, indicating that substitution was favoured at both the hydroxyl group and the nitrogen atom in the solasodine molecule.

2.3.2. Optimization of the HPLC separation:

Once it had been established that peaks A and B were the desired compounds, separation of the peaks on HPLC was optimized. Initially, a range of isocratic programmes was tested, rather than gradient elutions, since the column would then not require re-equilibration after each run, which would shorten the time required for each run, considerably. Peak shape and resolution were very good when peaks A and B were separated using 100 % acetonitrile (Fig. 8). Resolution (R) of two

peaks can be mathematically defined as:

$$R = V_2 - V_1 / 0.5 (W_1 + W_2)$$

where V is the distance from the origin, retention time or elution volume of the peaks and W is the peak width measured in equivalent units (JOHNSON and STEVENSON, 1978). It is recommended (FRIEDMAN and LEVIN, 1992) that R should have a value of at least one. Under the present HPLC conditions, R had a value of 6.095, indicating that the peaks were well-resolved. However, it was felt that it would be advantageous if they were retained on the column for slightly longer, to separate them further from the initial peaks eluting with the void volume. When using actual plant extracts, it was conceivable that the initial peaks would broaden considerably, thus masking peaks A and B at their present retention times. It was acknowledged by JONKER, KOOPS and HOOGENDOORN (1992) that excessive reduction of run time may increase the number of analyses possible, but may have repercussions for the accuracy of the analysis of complex samples.

The polarity of the solvent was therefore increased progressively, by the addition of increasing amounts of 0.2 M acetic acid buffered to pH 3.5 with triethylamine, in an attempt to retain the compounds on the column slightly longer. The solvent compositions are indicated in Fig. 9. As the polarity of the solvent increased, so the retention times of the peaks were increased, but the peak quality deteriorated, with the peaks appearing as "bumps" when an 80 % acetonitrile and 20 % buffered acid water mixture was used. From these results, a mixture of 90 % acetonitrile : 10 % buffered acid water was chosen for all subsequent experimentation.

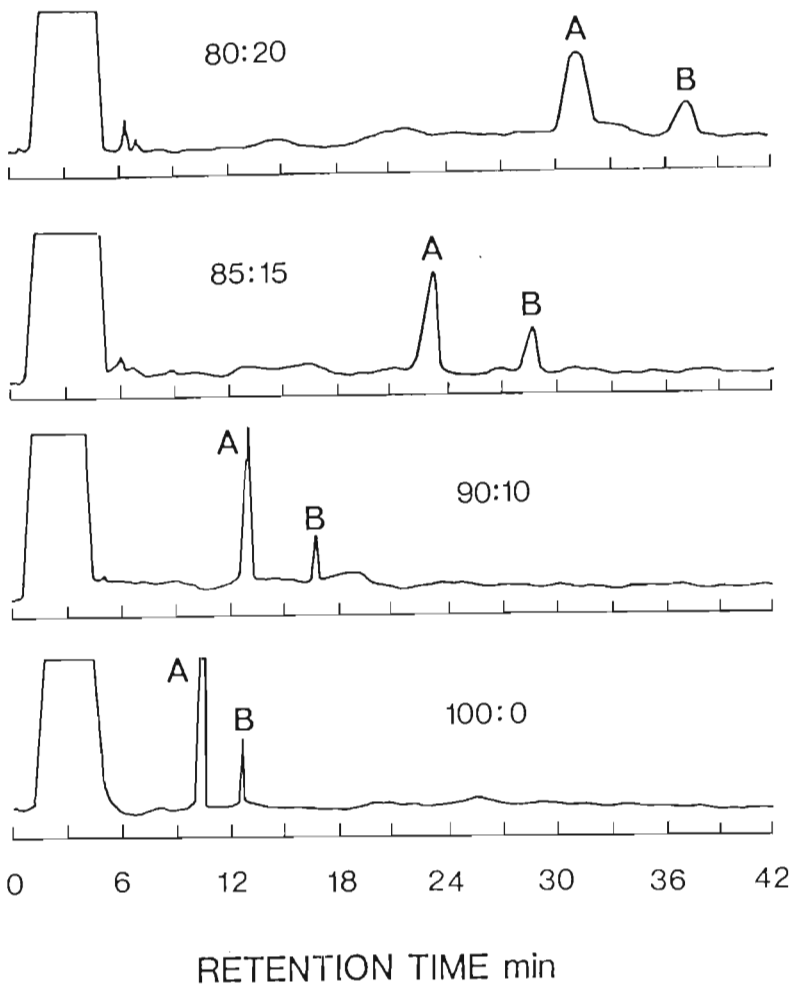

Figure 9. HPLC separation of benzoylated solasodine using a range of mixtures of acetonitrile : 0.2 M acetic acid buffered to pH 3.5 with triethylamine.

Under these conditions, peak shape was good, the peaks were well-resolved and the separation from the initial solvent peaks was sufficient. K', the capacity factor, gives an indication of the degree of retention of peaks. Small values of k' indicate that the components are little retained by the column and elute close to the void volume (JOHNSON and STEVENSON, 1978).

K' can be mathematically defined as:

$$k' = V_1 - V_0 / V_0$$

where V_1 is the distance, retention time or volume of the peak and V_0, the distance, retention time or volume of the unretained peak. CRABBE and FRYER (1980) indicated that for a practical separation, the capacity factor of the peaks should be between one and 10. When using either 100 % acetonitrile or 90 % acetonitrile : 10 % buffered acid water, the k' values for both peaks A and B were between one and 10, but when using the other mixtures, the values were beyond this range. Therefore, the 90 % : 10 % mixture of solvents resulted in greater retention of peaks, as desired, but still gave a practical separation. It was therefore not necessary to attempt gradient elution.

This HPLC separation method was tested on two other available HPLC systems, namely a Beckman System Gold with a scanning detector module 167 and a dual-pump programmable solvent module 126, and a Spectra Physics machine with an SP8700XR extended range L.C. pump and a Hewlett Packard series 1050 variable wavelength detector. In all cases, a Hypersil 5 ODS column was used, detection was at 254 nm and aliquots of the same sample were injected. Although retention times differed considerably on all three machines (Table 5), separation was achieved in all cases when a mixture of 90 % acetonitrile and 10 % buffered acid water was used as the elution solvent. The longer retention times of peaks A and B on the Beckman System Gold and Spectra Physics machines could be attributable to the use of a different Hypersil 5 ODS column and the different pump systems. The successful separation of benzoylated solasodine on a range of machines indicated that the method could easily be adapted for other instruments. For all subsequent

analyses, the Varian Model 5000 liquid chromatograph was used.

Table 5. Retention times obtained for benzoylated solasodine on different liquid chromatographs, when using 90 % acetonitrile and 10 % buffered acid water as the elution solvent mix.

HPLC used	Retention time (min)	
	Peak A	Peak B
Varian	12.9	16.5
Spectra Physics	15.6	20.1
Beckman	17.4	22.4

Three identical aliquots of a benzoylated solasodine sample were injected into the Varian HPLC, but detection was conducted at different wavelengths, namely 230, 254 or 280 nm. This was done in order to determine whether 254 nm was indeed the best wavelength to use, or whether another region of the UV spectrum might give greater peak areas and thus effectively increase the levels of solasodine that could be detected. It was found (Fig. 10) that while peak area was greater at 230 nm, there were also more other compounds that absorbed at this wavelength than at 254 or 280 nm, thus resulting in a more crowded spectrum. At 280 nm, there were few other absorbing compounds, but peak area was lower than at 254 nm. It was therefore decided that detection at 254 nm would give the best compromise between sensitivity and acceptable interference from other compounds, in addition to being the most commonly-used wavelength of UV detectors. Subsequently, detection for all analyses was conducted at 254 nm.

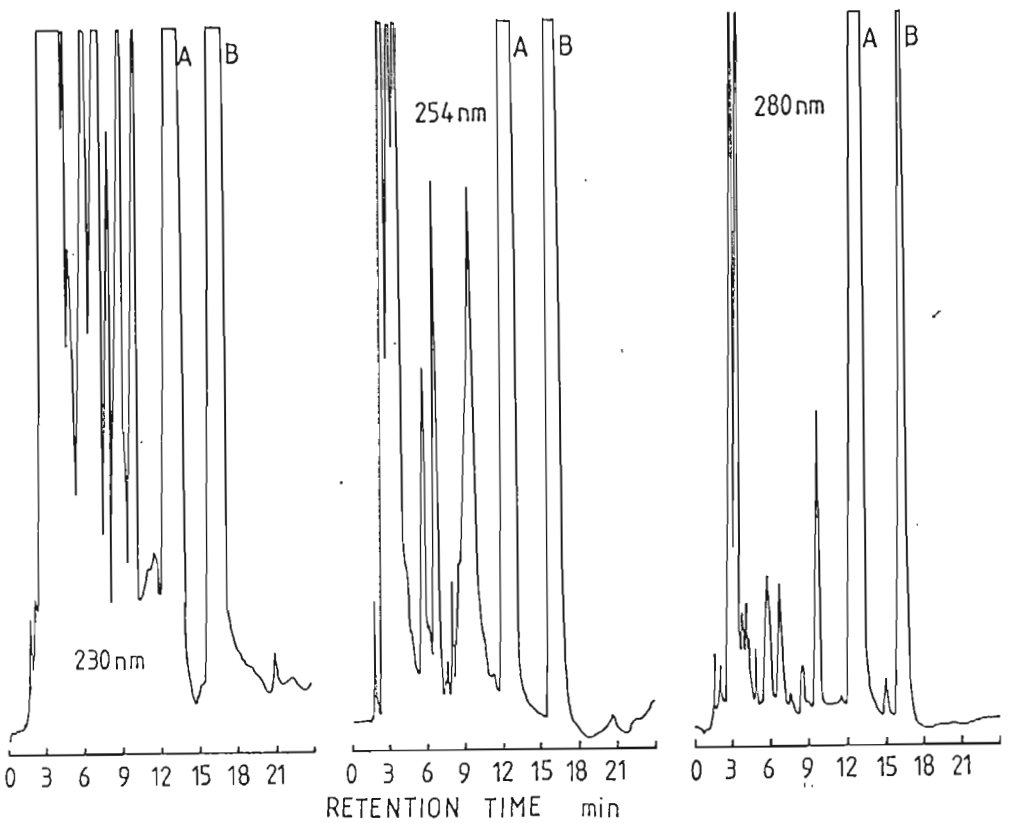

Figure 10. HPLC traces obtained when detection was at different wavelengths.

2.3.3. Characterization of benzoylated solasodine:

Throughout all experimentation, peak A (disubstituted solasodine) had a far greater area than peak B (monosubstituted solasodine), as mentioned previously (Section 2.3.2). However, the ratio of A and B was very inconsistent. When a random sample of twenty such ratios obtained from benzoylation trials conducted under a wide range of different conditions was averaged, a value of 5.094 $\pm$ 0.90 was

obtained. This indicates that, on average, there is five times more disubstituted solasodine formed than monosubstituted solasodine. However, if the reaction time was very short (30 minutes), the levels of the two products were sometimes almost equal. Presumably, the benzoylation reaction was not able to reach its usual equilibrium position in such a short time. At the other end of the scale, peak B sometimes disappeared altogether. No factor could be found that consistently resulted in the synthesis of disubstituted solasodine only. This would have been useful, since then peak areas would not have to be added in order to obtain an indication of the total amount of solasodine present. However, neither temperature, duration of the reaction, nor the amount of the reagents added appeared to control the position of the equilibrium of the benzoylation reaction. For example, a trial was conducted in which 2 mg samples of solasodine were dissolved in 1 ml of ethyl acetate and reacted with 8 μl benzoyl chloride with the addition of 6 μl of triethylamine for two, six, nine and 24 hours. The samples were initially warmed for five to ten seconds in a 50°C waterbath after which the reaction was allowed to proceed at room temperature. At the end of the reaction times, the reactions were terminated as described previously (Section 2.3.1) and the dried samples redissolved in 500 μl HPLC-grade methanol. Upon analysis by HPLC, it was found that both peaks A and B were present in the two, six and 24 hour samples, but peak B was absent in the nine hour sample. Similarly, when 1, 2, 3 and 5 mg samples of solasodine were allowed to react with 8 μl benzoyl chloride in the presence of 6 μl triethylamine for two hours, then processed as described above, the 1 and 5 mg samples yielded two peaks, and the 2 and 3 mg samples only peak A.

Throughout the duration of the experimentation with pure solasodine, periodic samples lacked peak B. Most samples, however, contained both peaks A and B. In a few cases, identical masses of solasodine were reacted concurrently and one sample ultimately yielded di- and monosubstituted solasodine while the other contained only the disubstituted product. In such cases, it was observed that peak area was slightly reduced (6 %) when only peak A was present, compared to the sum of the areas under A and B. This reduction was small in relation to the size of peak B and therefore acceptable.

It was thought that low levels of peak B and its occasional absence might be influenced by its lack of solubility in the injection solvent, namely methanol. Solasodine itself is only sparingly soluble in methanol. Therefore, a trial was set up in which 0.5 mg samples were dissolved in 1 ml of ethyl acetate and benzoylated with 9 μl benzoyl chloride. The samples were reacted for 2.5 hours at room temperature, before the reactions were terminated as described previously. After the final drying down of the samples under nitrogen, one was redissolved in 400 μl methanol and the other in 400 μl ethyl acetate. Twenty μl aliquots were injected into the HPLC. The results are given in Figure 11. In both cases, peaks A and B were present and of similar magnitude. Therefore, benzoylated solasodine appeared to be readily soluble in both methanol and ethyl acetate. In all further experimentation, HPLC-grade methanol was used as the final solvent.

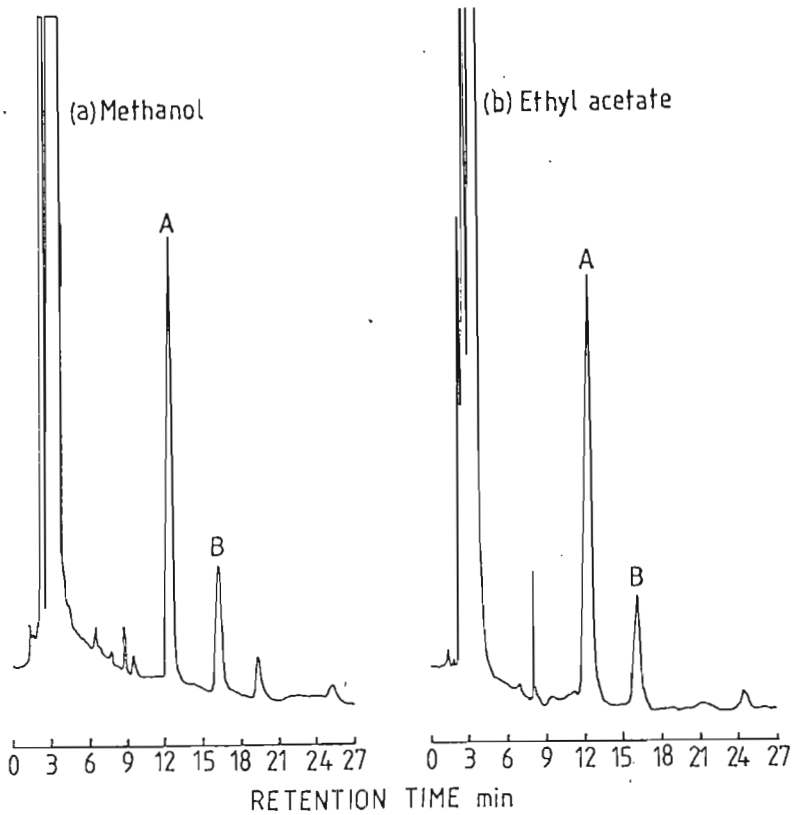

Figure 11. HPLC traces obtained when methanol (a) and ethyl acetate (b) were used as the final sample solvents.

Once the benzoylation reaction had been terminated by the addition of water, the levels of benzoylated products remained constant. This was tested by injecting aliquots of 1, 2 and 3 mg benzoylated solasodine samples at one weekly intervals for three weeks.

Table 6. Stability of benzoylated solasodine with time.

Time (days)	Peak area x 10^3		
	1 mg sample	2 mg sample	3 mg sample
0	3.15	5.68	7.84
7	3.17	5.68	7.48
14	3.29	5.65	7.17
21	3.26	5.48	7.21

Between injections, the samples were stored at room temperature in the light in airtight vials. The peak areas obtained are given in Table 6. Taking into account the lack of reproducibility due to injector error (Section 2.3.6), the slight fluctuations are negligible. Benzoylated solasodine is therefore very stable. In contrast, certain benzoylated polyamines, namely agmatine and spermidine, start to break down when stored beyond two weeks, even if kept at -20°C (FLORES and GALSTON, 1982).

2.3.4. Optimization of the benzoylation reaction:

The effect of a range of factors on the benzoylation reaction was tested in order to optimize the reaction. The optimal conditions would result in the greatest peak areas and thus, in effect, a greater level of detection. It was also borne in mind that the reaction time should not be too long, so that routine analyses could be done relatively quickly. The optimal conditions would therefore give the highest peak area in the shortest time.

Firstly, the effect of altering the amount of benzoyl chloride used for the reaction was tested. FITZPATRICK and SIGGIA (1973) routinely added a three molar excess when benzoylating animal steroids, but they did not ascertain whether or not this was, in fact, optimal. One mole of solasodine can react with two moles of benzoyl chloride, and therefore a minimum of two moles equivalent of benzoyl chloride would be necessary. However, an excess would be better, since this would shift the equilibrium of the benzoylation reaction such that as much of the solasodine present in the sample as possible, would react. For 1 mg of solasodine, a two molar equivalent of benzoyl chloride is 0.55 μl (molecular weight = 140; density = 1.21 g cm^{-3}) and of triethylamine, 0.67 μl (molecular weight = 101; density = 0.73 g cm^{-3}). All molar excesses were calculated relative to this.

In the first trial, 5 mg of solasodine was dissolved in 5 ml of ethyl acetate. One ml (ie: 1 mg) samples were removed into four other tapered glass vials. A molar equivalent (ie: twice as much benzoyl chloride as solasodine on a molar basis), or a 2, 3, 4 or 5 M excess of benzoyl chloride and triethylamine were added to the samples. The vials were warmed for five to ten seconds in a 50°C waterbath to accelerate the reaction, which was then left to proceed for 2.5 hours at room temperature. Distilled water was subsequently added to terminate the reaction and the ethyl acetate layer washed three times with distilled water before being dried over sodium sulphate. The ethyl acetate fractions were dried down under nitrogen and redissolved in 300 μl HPLC-grade methanol prior to injecting into the HPLC.

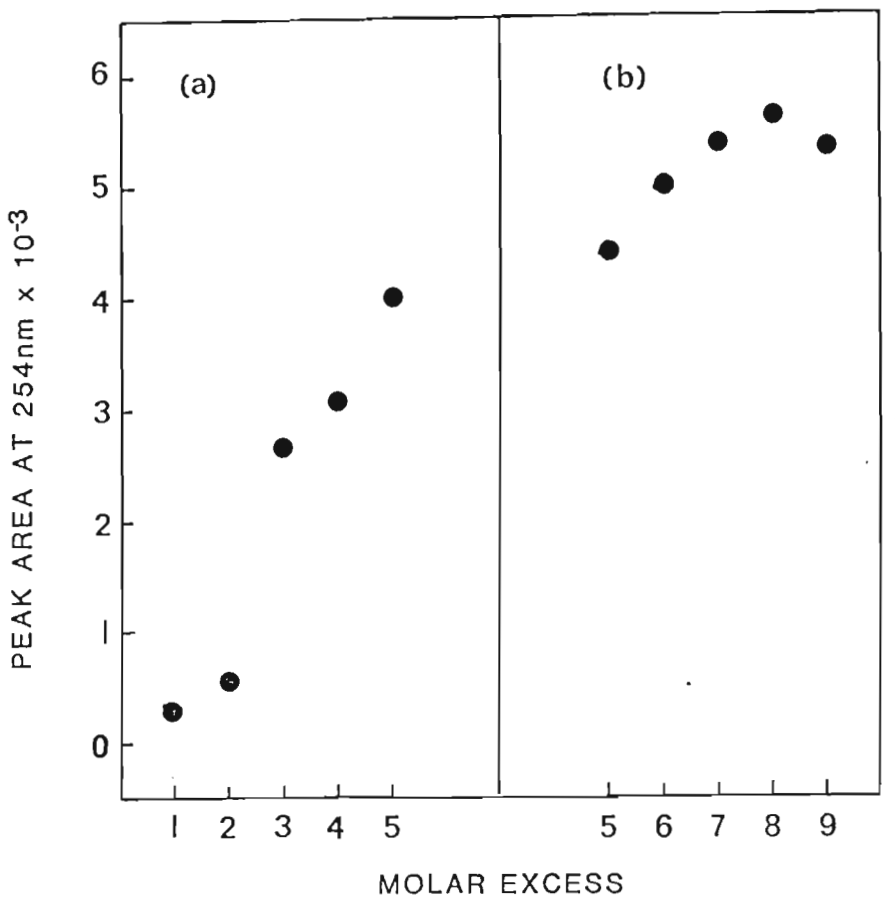

Figure 12. HPLC peak areas (A + B) obtained for benzoylated solasodine when using increasing amounts of benzoyl chloride.

With an increase in the excess of the reagents, the peak area increased (Fig. 12a) and this still appeared to be the trend with a 5 M excess of benzoyl chloride. The reaction was apparently not yet saturated in the time allowed for the reaction. Therefore, the trial was extended to include a 5, 6, 7, 8 and 9 M excess of benzoyl chloride and triethylamine. Again, 1 mg samples were used and treated as described in the first part of the trial. At this level, the increase in peak area with increase in molar excess of reagents was not nearly as great (Fig. 12b) and appeared to stabilize when a 6 to 7 M excess was used. This indicated that any further increase in the amount of benzoyl chloride added would not result in an

increase in peak area, under these experimental conditions and would therefore give no added advantage. The trial was repeated using 2 mg solasodine samples with a 2, 4, 6 or 8 M excess. Again, the increase in peak area tailed off at a 6 M excess.

This then led to the question of how much reagent one should add to a sample containing an unknown amount of solasodine, in order to obtain an accurate result. A trial was therefore set up in which the same amount of reagent was added to different masses of solasodine. The masses used were 1, 2, 3 and 5 mg of solasodine and these samples were reacted with 3 μl benzoyl chloride and 3.6 μl triethylamine, which is a 6 M excess for 1 mg of solasodine. Thus, as the mass of the samples increased, so the degree of excess of the reagents decreased. After initially warming the samples in a 50°C waterbath for five to ten seconds, the samples were left to react at room temperature for 2.5 hours. At the end of this time period, the reactions were stopped with distilled water and the samples processed as described for the previous trials. Aliquots (30 μl) of the samples were then injected into the HPLC. It was found (Fig. 13) that although the degree of reagent excess decreased, peak area still increased significantly with an increase in mass. Therefore, although the reaction may not be saturated in all cases and so the calculation of the absolute amount of solasodine not fully accurate, relative differences in solasodine are still obvious. If the absolute amount of solasodine present in a set of samples is required, the researcher should react several samples with increasing amounts of benzoyl chloride, to find the level at which the reaction is saturated.

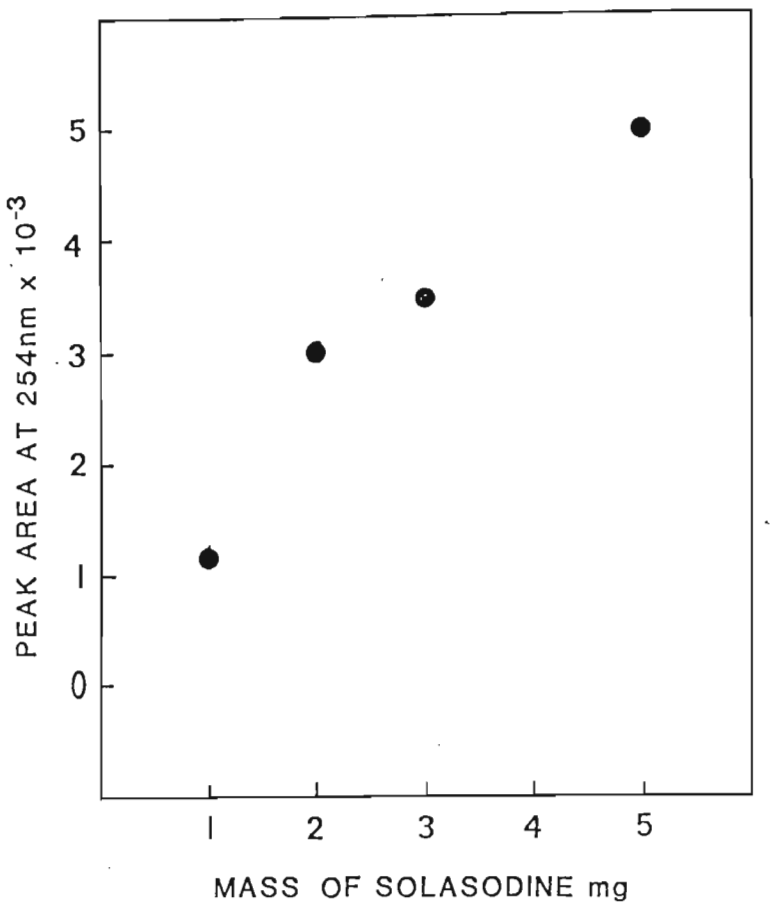

Figure 13. HPLC peak area (A + B) obtained when increasing masses of solasodine were reacted with the same amount of benzoyl chloride.

In general, however, when working with a familiar plant system, levels of reagent can be used that would saturate the reaction when the maximum expected level of solasodine is present.

The second factor in the benzoylation reaction that was examined was the duration of the reaction. Prior to this, a reaction time of 2.5 hours or less had been used, with a few seconds of heating at the start of the reaction. It was necessary to determine whether, after 2.5 hours, the benzoylation reaction was still proceeding, or whether it had reached equilibrium. If not, an increase in reaction time could result in greater peak area, as the reaction proceeded further, and thus an increase in overall sensitivity of the method. If the reaction were saturated sooner than 2.5

hours, the reaction time could be reduced. This was a distinct possibility, since FLORES and GALSTON (1982) allowed the benzoylation of polyamines to proceed for only 20 minutes, at room temperature.

A 5 mg sample of solasodine was weighed into a tapered glass tube and dissolved in 5 ml ethyl acetate. Subsamples (1 ml each) were then transferred to four other glass tubes. A 6 M excess of benzoyl chloride (3μl) and triethylamine (3.5 μl) was added. The samples were warmed briefly (five to ten seconds) in a 50°C waterbath and then left to react at room temperature for 1, 2.5, 6, 24 and 48 hours. At the end of the incubation periods, the reactions were stopped with water, the samples washed and then dried over sodium sulphate, as described previously. The ethyl acetate fractions were decanted from the sodium sulphate residue into airtight vials, dried down under nitrogen and redissolved in 300 μl HPLC-grade methanol. They were then injected into the HPLC.

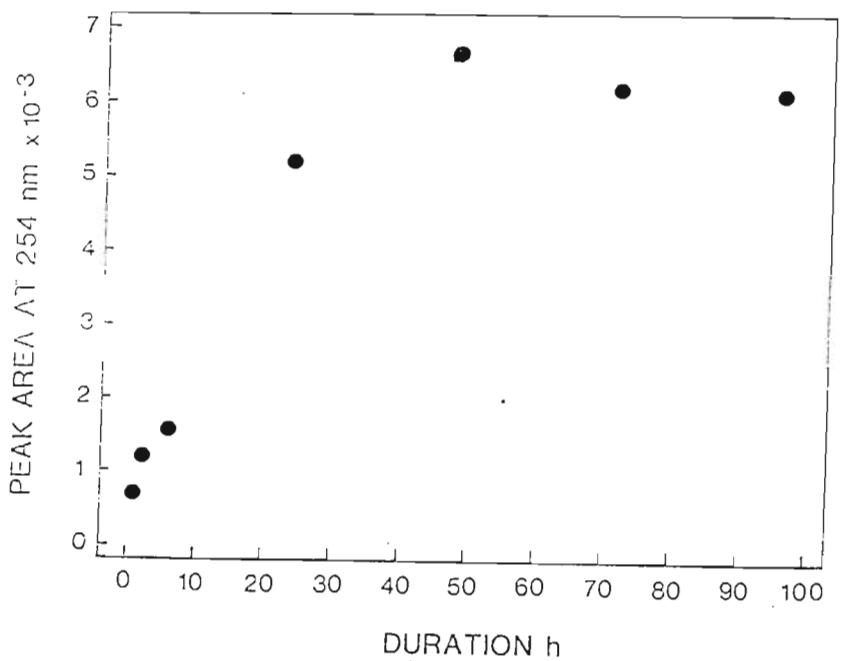

Figure 14. HPLC peak areas (A + B) obtained following the reaction of solasodine with a 6 M excess of benzoyl chloride for increasing time periods.

It was observed (Fig. 14) that initially (1 - 24 hours), peak area increased greatly with increasing reaction time, but this increase was not quite as marked beyond this time.

In order to pinpoint the cut-off time at which the reaction would proceed no further in this direction, the trial was extended to include reaction times of 72 and 96 hours. These samples, together with fresh 24 and 48 hour samples, were treated as described in the first part of the trial. It was found (Fig. 14) that saturation point had indeed been reached just after the 24 hour reaction time, the peak area no longer increasing significantly if the reaction time was extended. Thus, it was established that the maximum level of detection was attained between 24 and 48 hours.

This is far longer than the 20 minutes used for the benzoylation of polyamines (FLORES and GALSTON, 1982), but these authors did not indicate whether or not they had tested to see if this was the optimal reaction time. The lengthy time period required for full solasodine benzoylation is, however, not very practicable if results need to be obtained rapidly. The options therefore, were either to choose a specific reaction time and use it throughout, or to accelerate the reaction by some means.

One factor that could influence the rate of the benzoylation reaction, is temperature. FITZPATRICK and SIGGIA (1973) benzoylated animal steroids from urine and blood samples at 80°C and indicated the reaction to be complete after 15 min. However, since solasodine is known to be slightly heat sensitive (LANCASTER and MANN, 1975), we chose to elevate the temperature to only 50°C.

The effect of heating the samples for a longer part of the entire reaction time than the few seconds used in previous trials, was tested. A 7 mg sample of solasodine was dissolved in 7 ml ethyl acetate, then split into seven 1 ml samples, each containing 1 mg of solasodine. After the addition of a 6 M excess of benzoyl chloride and triethylamine, the glass reaction vessels were placed in a 50°C waterbath for 0, 30, 60, 120 minutes, 4, 6 or 8 hours. At the end of the heating times, the samples were removed to room temperature for the remainder of the reaction time. Thus, all samples had a total reaction time of 8 hours, so that any differences in peak area subsequently observed, would be as a result of the different temperature treatments.

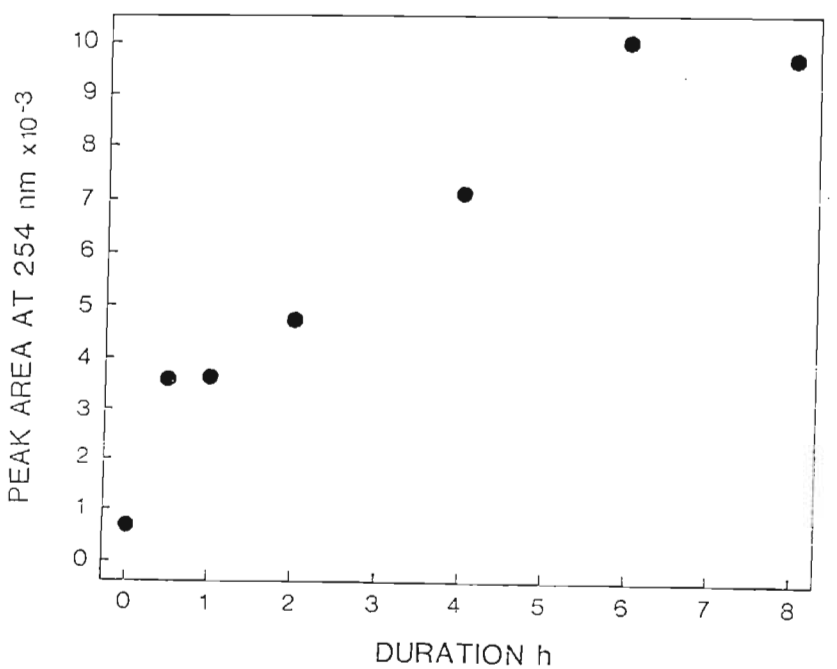

Figure 15. HPLC peak areas (A + B) obtained following reaction of solasodine with a 6 M excess of benzoyl chloride for a total of 8 hours, with heating at 50°C for various time periods up to 8 hours.

Heating was found to affect the reaction profoundly (Fig. 15). Maximum peak area was observed after 6 hours of heating, whereafter there was little difference in peak area. When heated for a short while (zero to two hours), peak area was very much lower and it increased steadily with increased duration of heating. In these instances, a longer total reaction time would be necessary to reach an equivalent peak area. Therefore, it was concluded that a 6 hour heating treatment was optimal for the benzoylation to proceed to its fullest extent. This was far more time efficient than the 24 to 48 hour period required if the reaction vessel was only warmed for a few seconds at the commencement of the reaction. Overall, therefore, optimal benzoylation was concluded to occur when a 6 M excess of benzoyl chloride was used and the reaction was allowed to proceed for 6 hours at 50°C.

2.3.5. Use of the optimized benzoylation reaction:

Once the reaction had been optimized, samples could be reacted under the appropriate conditions and used to establish certain important facts. Firstly, it was necessary to determine whether or not detection of benzoylated solasodine was linear over a useful mass range, such that a standard curve could be constructed. A 1.2 mg sample of solasodine was weighed out, dissolved in ethyl acetate and reacted with a 6 M excess of benzoyl chloride. The sample was left to react for six hours at 50°C, then processed in the usual manner. Finally, the sample was redissolved in 320 μl HPLC-grade methanol. Six aliquots, ranging from 10 to 100 μl, were then injected into the HPLC. The increasing volumes corresponded to

increasing amounts of benzoylated solasodine. The mass equivalent of solasodine in each sample could be calculated as a proportion of the original 1.2 mg used. From this data, a standard curve was constructed (Fig. 16).

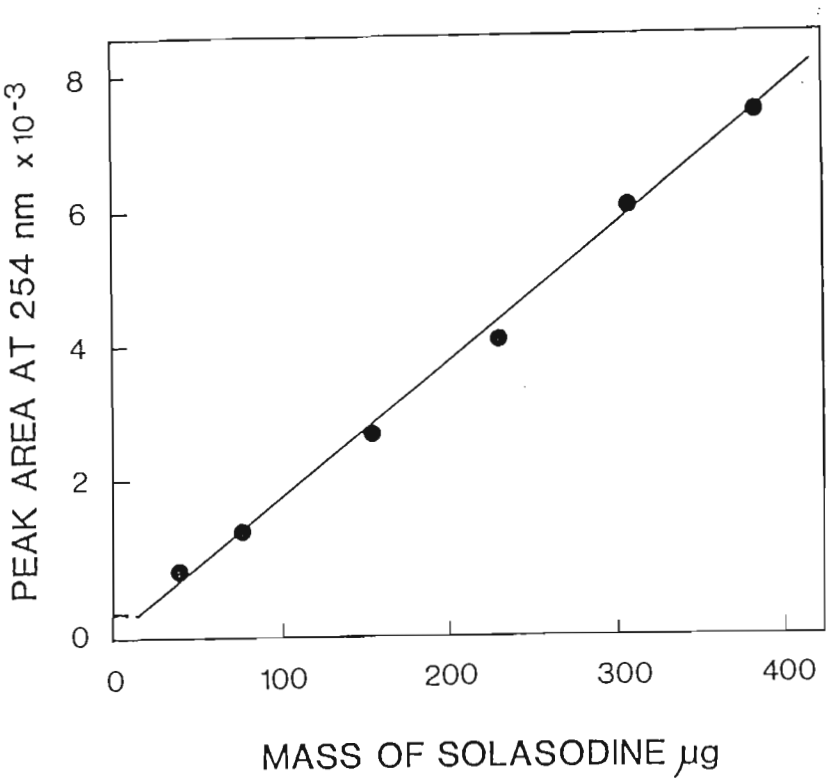

Figure 16. Standard curve obtained for solasodine following the injection of various quantities of benzoylated solasodine. The benzoylated solasodine was obtained by reacting solasodine with a 6 M excess of benzoyl chloride for 6 hours at 50°C.

Over the range of masses tested, namely from 30 to 400 μg, a linear relationship was observed with a correlation coefficient of 0.9980. From such a curve, unknown masses of solasodine in plant extracts could then be determined. MANN (1978) indicated the content of solasodine in *Solanum* species to range between zero and five percent, on a dry weight basis. Therefore, if 0.5 g of dried plant material

is extracted, solasodine should be detectable when using this technique, even if low levels are present. This is assuming that there is no loss during extraction. In cultured tissues, such as callus, where levels of secondary products are usually lower than in the parent plants (ANDERSON, PHILLIPSON and ROBERTS, 1986), sample masses probably should be increased further, to ensure accuracy. The method developed thus appeared to be suitable for the detection and quantification of solasodine in plants by HPLC.

The second important fact that could now be determined was the minimum level of detection that could be achieved using the outlined method. A 1 mg sample of solasodine was benzoylated under optimal conditions and processed as described. When a 1 μl aliquot, which corresponded to 3.5 μg of solasodine, was injected, benzoylated solasodine could still be visualized as two distinct peaks, although peak B was becoming very small. Detection is therefore easily down to the microgram level, when the HPLC is set at 0.05 absorbance units full scale (AUFS) and an attenuation of 16.

2.3.6. Reproducibility:

An important factor to determine when setting up a new technique, is the variability. This enables the researcher to predict whether a difference is true, or whether it can simply be ascribed to experimental error. Firstly, the reproducibility between injections was determined. Four samples from the heating trial (Section 2.3.4), containing increasing amounts of benzoylated solasodine, were each injected three times in succession, and the average peak areas and their standard deviations

calculated. The variability was found to range from 0.8 to 2.2 % of the mean. The larger percentage variation occurred when samples containing low amounts of benzoylated solasodine were injected. This error is due to the human factor involved and cannot be reduced. It was then tested to see how variable results were when equal amounts of solasodine were benzoylated simultaneously. Three 1 mg samples were used for this purpose and benzoylated under optimal conditions. Resultant error was found to be 6.1 % of the mean. This would be due to differences in initial weighing of the samples, differences in the degree of completion of the reaction, transfer losses and would include the injection error described previously. This overall variability was less than 10 % of the mean and was therefore regarded as acceptable. It was noted throughout the duration of experimentation, however, that samples of the same mass, benzoylated at different times, could yield considerably different peak areas, presumably due to slight differences in benzoylation conditions. It was therefore recommended, that at least three external standards are run with each batch of samples that is benzoylated, in order to ensure accuracy.

2.4. Conclusion

After the initial HPLC detection and identification of benzoylated solasodine, much experimentation was required in order to optimize both the HPLC separation and the benzoylation process. Ultimately, it was found that an isocratic program consisting of 90 % acetonitrile and 10 % buffered acid water (pH 3.5) fulfilled all the requirements. For the benzoylation reaction, a 6 M excess of benzoyl chloride was found to be sufficient to allow the reaction to proceed as far as possible. At

an elevated temperature of 50°C, the optimal reaction time was found to be 6 hours. For the remainder of the experimentation, which involved finding a suitable extraction technique and applying it to plant material, these conditions were strictly adhered to.

CHAPTER 3

EXTRACTION AND PURIFICATION OF SOLASODINE

3.1. Introduction

In the work described in Chapter 2, in which solasodine was derivatized and an HPLC technique developed for its detection, authentic solasodine was used. However, it is a very different prospect when solasodine from plant material is analyzed. In the latter situation, the compound must be extracted from the plant matrix and then purified, until the extract is sufficiently pure to allow for HPLC determination of solasodine. If the extract is not pure and other compounds are present that have similar retention times to benzoylated solasodine, when separated on HPLC, they may obscure its presence. Therefore, an extraction and purification protocol was established that would complement the benzoylation and HPLC detection techniques being used. MACÉK (1989) is of the opinion that the method of extraction is almost as important as the analysis itself.

A variety of techniques exists for the extraction of glycoalkaloids. Prior to extraction, however, the material is usually dried in some way, since this apparently stabilizes the solasodine content of the plant material (CRABBE and FRYER, 1982). LANCASTER and MANN (1975) found that in *Solanum laciniatum* Ait., much solasodine was lost within 12 hours at oven temperatures of 105°C and above and therefore recommended that temperatures below 100°C be used for drying. An alternative is to freeze-dry the material, if this option is available, prior to grinding.

Initial extraction usually involves the use of one of three solvents:

i) dilute aqueous acids;

ii) alcohols; or

iii) acidified alcohols.

The first option is widely used. The acid of choice is usually two to five percent acetic acid (CHAUDHURI and CHATTERJEE, 1979; TELEK, 1979; EMKE and EILERT, 1986; CHAM and WILSON, 1987; HERBRETEAU-LEMONNIER, ROSSIGNOL, ROSSIGNOL, SIHACHAKR and HAICOUR, 1989), although sulphuric (BEZBURUAH, 1981) and oxalic (LANCASTER and MANN, 1975) acids are used in some instances. Completely different acid systems are used by researchers extracting glycoalkaloids from potatoes and commercial potato products, namely tetrahydrofuran-water-acetonitrile in 1 % acetic acid (BUSHWAY, BUREAU and KING, 1986; FRIEDMAN and LEVIN, 1992) or water containing 1-heptanesulphonic and acetic acids (CARMAN, KUAN, WARE, FRANCIS and KIRSCHENHEUTER, 1986). The second solvent option, the use of alcohols, also seems traditionally to have had wide appeal. Either ethanol (HOSODA, ITO and YATAZAWA, 1979; BHATT, BHATT and SUSSEX, 1983; PATIL and LALORAYA, 1984) or methanol (CRABBE and FRYER, 1980; WEILER, KRÜGER and ZENK, 1980; GUPTA and BASU, 1981; QUYEN, KHOI, SUONG, SCHREIBER and RIPPERGER, 1987; RIPPERGER and PORZEL, 1992) are used, at concentrations of 70 to 100 %, at room temperature or with heating at about 60°C. Alcohol extracts yield more total alkaloid than dilute aqueous acid extracts, but are significantly more contaminated with other compounds such as pigments and

resins, and thus require more purification. Losses during this process can apparently result in yields comparable to those of aqueous acid extraction methods (MANN, 1978). TELEK (1979) reported that aqueous acid extracts of *Solanum mammosum* L. and *S. khasianum* Clarke fruits contain a heavy suspension of colloidal pectin, which has to be removed by precipitation with ethyl acetate. Both of the two extraction solvent options mentioned, therefore, have certain difficulties associated with them, and the method chosen seems to be a matter of personal preference. The third option is a combination of these two methods, namely the use of acidified alcohols. Hydrochloric (PANDEYA, SARATBABU and BHATT, 1981b; CARLE and REINHARD, 1980) or, more usually, two to five percent acetic (KADKADE, RECINÓS and MADRID, 1979; JAIN and SAHOO, 1981a; 1986; RODDICK and MELCHERS, 1985; RIDOUT, PRICE, COXON and FENWICK, 1989; LAWSON, ERB and MILLER, 1992) acids are used in either ethanol or methanol.

Prior to or following the initial extraction, the extracts may be defatted to remove very non-polar fats and waxes that may cause problems during subsequent partitioning due to the formation of emulsions (CORDELL, 1981). This removal is accomplished using petroleum ether (VÁGÚJFALVI, MARÓTI and TÉTÉNYI, 1971; PATIL and LALORAYA, 1984), chloroform (CAMPBELL, 1990), hexane:ether (1:1) (RIPPERGER and PORZEL, 1992) or benzene:ether (1:1) (KHANNA, UDDIN, SHARMA, MANOT and RATHORE, 1976).

Normally, glycoalkaloids are analyzed as their aglycones, for example solasodine, since hydrolysis is a useful purification step (CRABBE and FRYER, 1982). Some

methods combine the extraction and hydrolysis step into a single direct hydrolysis (PANDEYA, SARATBABU and BHATT, 1981b), but loss of solasodine may occur during this process (CRABBE and FRYER, 1982). Generally, therefore, hydrolysis is carried out at some stage subsequent to extraction. An undesirable side-reaction during hydrolysis leads to the formation of solasodiene, by the removal of the C-3 hydroxyl group. To minimize solasodiene formation, it has been recommended that the hydrolysis be carried out under reflux with alcohol-water mixtures, using hydrochloric or sulphuric acids. The solasodine aglycone is insoluble in water and the alcohol keeps it in solution (MANN, 1978). Alternatively, it has been suggested that glycoalkaloids can be microbiologically hydrolyzed using *Aspergillus niger* (LAHA and BASU, 1983).

After acid hydrolysis, the mixture is generally made alkaline, by the addition of sodium carbonate (CORDELL, 1981), sodium hydroxide (LANCASTER and MANN, 1975; CAMPBELL, 1990) or, more usually, ammonia (AHMAD, ALI and AHMAD, 1980; PATIL and LALORAYA, 1984; LAWSON, ERB and MILLER, 1992). In some instances, however, use of ammonia may give rise to new alkaloids not present in the original plant (CORDELL, 1981). The basification of the extract results in the precipitation of alkaloids such as solasodine, which can then be removed as a solid, or extracted into organic solvents such as benzene, chloroform or xylol (MANN, 1978). In some instances, analysis may then proceed, but frequently, a form of chromatographic separation may need to be introduced to make the isolation procedure more specific, such as paper, thin-layer, ion-exchange (CRABBE and FRYER, 1982) or silica gel (RIDOUT, PRICE, COXON and FENWICK, 1989) chromatography. An option that is increasing in popularity is

the use of solid-phase extraction (SPE) as a single purification step, following initial extraction (CRABBE and FRYER, 1980; BUSHWAY, BUREAU and KING, 1986; CARMAN, KUAN, WARE, FRANCIS and KIRSCHENHEUTER, 1986; VOGEL, JATISATIENR and BAUER, 1990; JONKER, KOOPS and HOOGENDOORN, 1992).

The described procedures are the basis of most techniques used in the analysis of glycoalkaloids and alkaloids. The variations on the basic theme are, however, innumerable, as each researcher has introduced modifications to suit specific requirements. This can conceivably lead to the confusion of researchers wishing to enter this field. The best option is to follow the basic protocol and to test which modifications fulfil particular requirements. This is the approach adopted in the present study.

3.2. Materials and Methods

The plant material used for the development of the extraction and purification procedures was berry material of *Solanum mauritianum* Scop. The green berries were all collected in the same locality (Muswell Hill, Pietermaritzburg). They were flash-frozen in liquid nitrogen to crack them open, freeze-dried, then ground to a powder in a Thomas-Wiley intermediate mill, using an integral gauze of 40 mesh. All other plant material ultimately used to test the extraction and purification procedures, was treated in the same manner.

For thin layer chromatographic analysis during the development procedure, 0.2 mm

plastic-coated silica gel 60 F_{254} plates were used. These were cut into 10 cm high plates, with the width depending on tank size and the number of samples to be analyzed at any particular time. The plates were developed in glass tanks using an acetone:hexane (1:1) solvent mixture. After a brief air-drying, the plates were dipped into a cobalt thiocyanate alkaloid staining solution (ZWEIG and SHERMA, 1972). This was made up by dissolving 3 g ammonium thiocyanate and 1 g cobalt(II)-chloride in 20 ml of distilled water. With this stain, alkaloids become clearly visible as blue or green spots on a pink background.

The benzoylation and HPLC conditions and procedures used throughout this chapter, are those that were concluded to be optimal in Chapter 2.

3.3. Method Development and Discussion

3.3.1. Initial extraction procedure:

Using the basic procedure outlined in Figure 17, the extraction of a 5 g of freeze-dried *Solanum mauritianum* berries was attempted.

The samples were extracted twice with ethanol to ensure complete extraction of the glycoalkaloids. It has been reported (CRABBE and FRYER, 1982) that the amount of solasodine obtained from the second extraction may be 30 to 40 % of that of the first extraction. However, the more finely ground the material is, the more complete the first extraction. CHAM and WILSON (1987) indicated that at room temperature, a minimum of four hours is required for complete glycoalkaloid

extraction, therefore, an extraction time of three hours in total, at an elevated temperature, was considered sufficient for the present study.

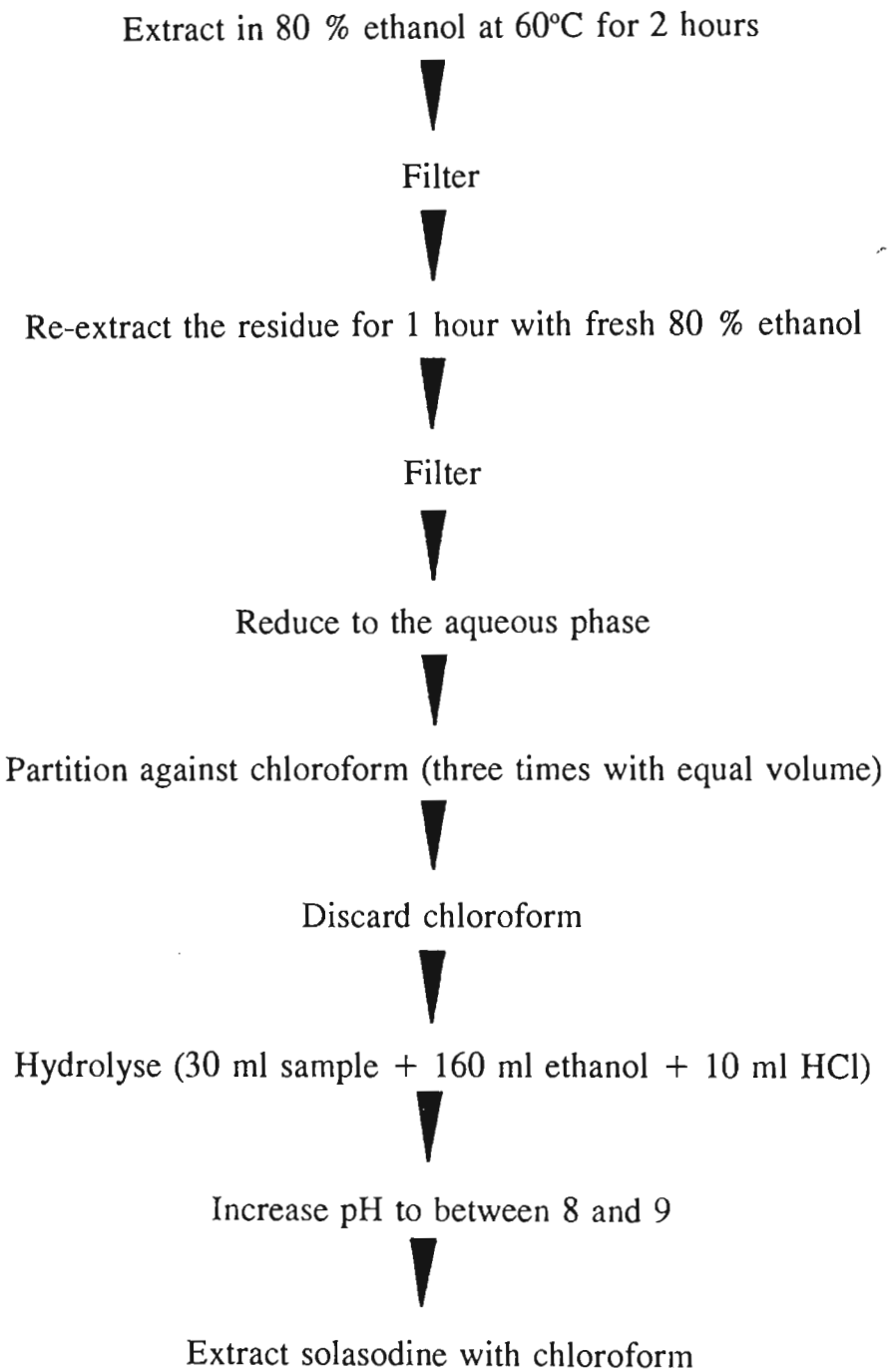

Figure 17. Basic extraction procedure initially followed for solasodine isolation.

After two partitionings of the extract against chloroform to remove lipids and pigments, no more coloured compounds were removed, the third chloroform fraction remaining clear. The extract itself was now a golden brown colour. Since some authors use petroleum ether rather than chloroform (VÁGÚJFALVI, MARÓTI and TÉTÉNYI, 1971), its efficacy was also tested. At least four partitionings with petroleum ether seemed to be required in order to remove all pigments. Chloroform was therefore subsequently used. A few species of *Solanum*, such as *Solanum viarum* Dun. (PATIL and LALORAYA, 1984), are reported to contain some solasodine in the free, rather than the glucosylated form. This would be extracted in the chloroform at this stage, since free solasodine is highly soluble in this solvent. Therefore, the three chloroform fractions from the partitioning were combined and the resultant sample taken to dryness as far as possible. A fraction of this sample was spotted onto a TLC plate and treated as described. No spot of solasodine was apparent, indicating a lack of free solasodine in *S. mauritianum*. Chloroform was thus used for this step in all subsequent analyses.

The hydrolysis of solasodine was conducted under reflux for two hours. The amount of hydrochloric acid approximates to a concentration of 0.5 M. This is the concentration recommended by LANCASTER and MANN (1975), who found that hydrolysis with 2 M hydrochloric acid for 90 minutes caused almost complete loss of solasodine, as solasodiene. The duration of hydrolysis that they subsequently recommended was one hour, whereas CRABBE and FRYER (1982) indicated five hours to be optimal. A hydrolysis time of two hours was decided upon in the present study. Following hydrolysis, the ethanol was removed under vacuum and

the pH then increased to between eight and nine, by the addition of 25 % ammonium hydroxide. This was done as quickly as possible, since the removal of the ethanol results in an increase in the concentration of the acid, which is not desirable. The precipitated solasodine was then extracted three times into chloroform. The three fractions were combined and taken to dryness, whereafter the sample was benzoylated and an aliquot injected into the HPLC. The resultant trace is given in Figure 18.

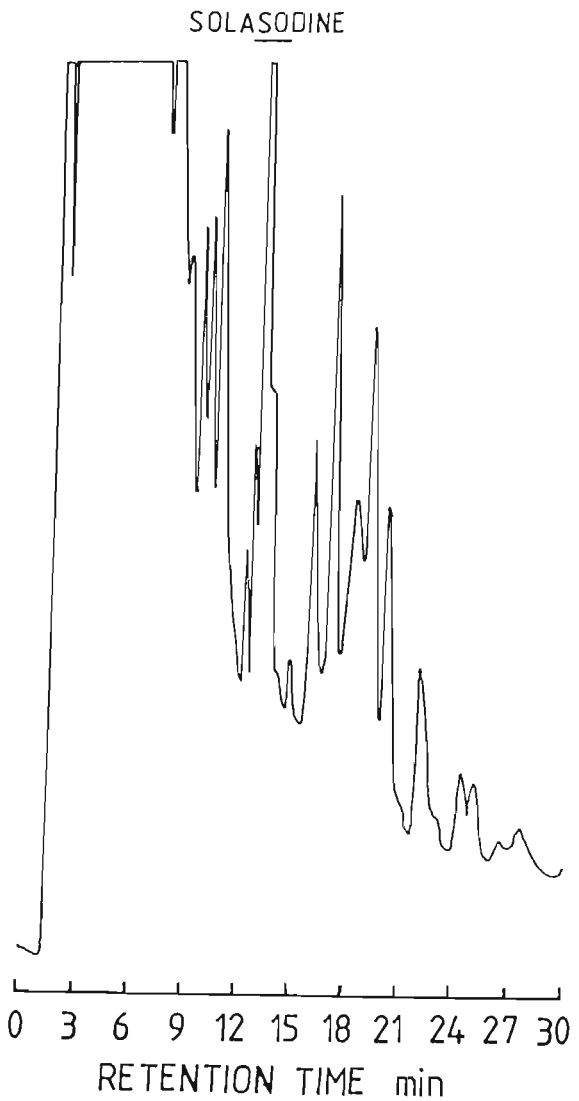

Figure 18. HPLC trace obtained following the extraction of 5 g of freeze-dried green *S. mauritianum* berries, using the basic procedure outlined in Figure 17. The retention time of authentic benzoylated solasodine is indicated.

Although it seemed that solasodine might be present, since peaks were present with retention times similar to that of the benzoylated solasodine standard, the trace was unsatisfactory, with peak resolution being very poor. Further purification definitely seemed to be necessary, in order to isolate solasodine. Perhaps the other compounds present interfered with the benzoylation of solasodine, thus preventing it rising above the background peaks and becoming easily detectable. Considering the sensitivity of the HPLC detection method (Chapter 2.3.5) and previously recorded levels of solasodine in solanaceous species (Chapter 1.2.3) solasodine should be easily detectable in a 5 g sample (dry weight).

The final sample that was injected into the HPLC was a clear brown colour. A simple purification step, namely the addition of charcoal was therefore attempted, in order to clarify the solution. This resulted in a clear solution that, upon injection yielded a very clean trace, with only the initial solvent peaks remaining. It was therefore concluded that both solasodine and the interfering impurities were adsorbed onto the charcoal, and this method of purification was abandoned. An alternative purification technique was therefore sought.

3.3.2. *Silica gel flash chromatography*:

On the recommendation of the Chemistry Department, University of Natal, Pietermaritzburg, a silica gel chromatographic step was introduced. Such a step has recently been used by some other researchers attempting to purify glycoalkaloid samples (RIDOUT, PRICE, COXON and FENWICK, 1989; RIPPERGER and PORZEL, 1991; 1992; LAWSON, ERB and MILLER, 1992). A form of

chromatography known as flash chromatography (STILL, KAHN and MITRA, 1978) was used. In this process, rapid separation, with moderate resolution, is obtained by driving the appropriate solvents through a relatively short column, under pressure. The silica gel of choice is 40 to 63 μm (400 to 230 mesh) (Merck) and a pressure-driven flow rate of 5 cm per minute is desirable. The method allows separation of samples weighing 0.01 to 10 g in 10 to 15 minutes (STILL, KAHN and MITRA, 1978). The apparatus, which is 40 cm long and has a diameter of 3 cm, is shown in Figure 19.

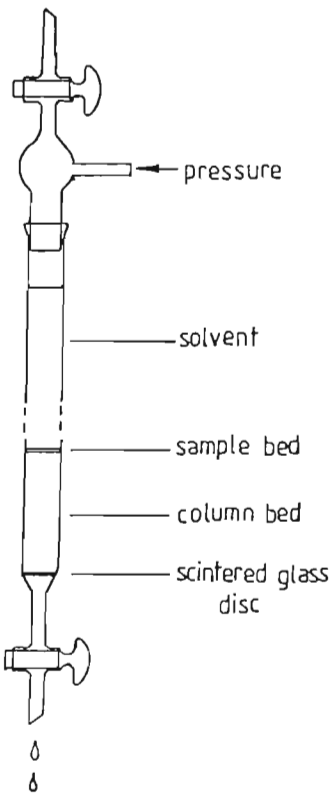

Figure 19. Apparatus used for silica gel flash column chromatography.

A 15 cm column (about 45 g of silica gel) was tested. A 5 g freeze-dried sample of *Solanum mauritianum* berry material was extracted as described, but, when

drying down the chloroform fraction under vacuum, just prior to benzoylation, 1 g of silica gel was added to the flask. By removing the remaining solvent under vacuum, the sample became adsorbed onto the silica gel. After sonication, the silica gel plus sample could easily be removed from the flask, ready to load onto the column.

The column was prepared by making a slurry of silica gel in hexane, a solvent in which solasodine is insoluble. The column was tapped to release any airbubbles and the column bed settled by passing through 100 ml of hexane, under pressure. When the level of solvent was just above the column bed, the sample was added and washed down the sides of the column with hexane to form a uniform sample bed. A 3 cm filter paper disc was floated down onto the sample bed to reduce disturbance thereof when adding solvent.

An additional 200 ml of hexane was then passed through the prepared column, followed by 500 ml of 20 % acetone in hexane, in which the solasodine should elute. Finally, 200 ml of pure acetone was washed through the column.

CAMPBELL (1990) reports another alkaloid to elute in the last fraction, but this was not examined in the present study. The 20 % acetone in hexane fraction was collected and dried down under vacuum before being benzoylated in preparation for HPLC analysis. A more distinct solasodine peak was now present (Fig. 20) that was better resolved from neighbouring, interfering peaks than before (Fig. 18). The use of the silica gel purification step therefore seemed to be distinctly advantageous.

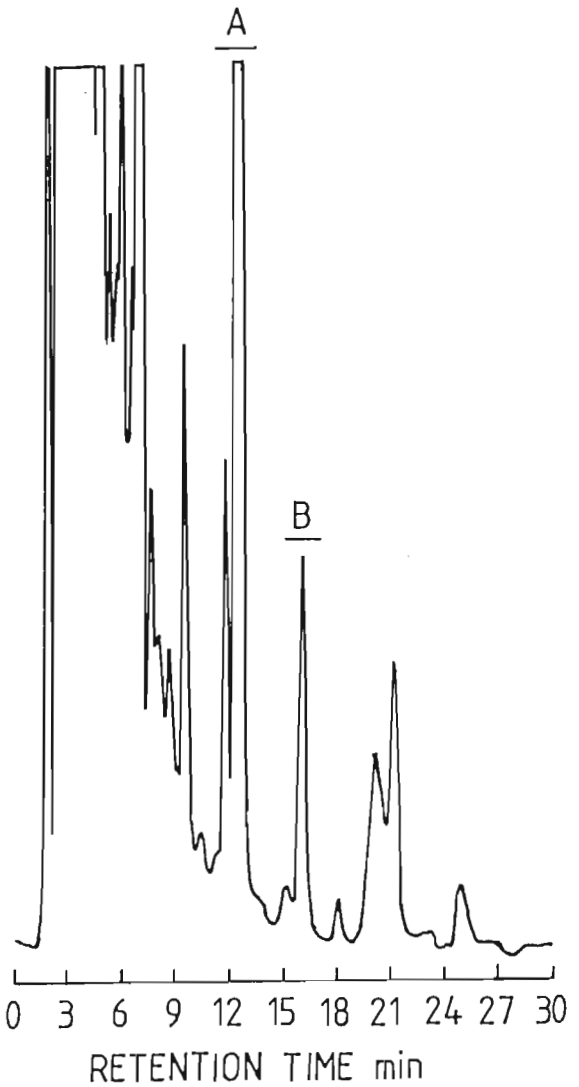

Figure 20. HPLC trace obtained following extraction of 5 g of freeze-dried *S. mauritianum* berry material and purification by silica gel column chromatography. A indicates the retention time of dibenzoylated solasodine and B, that of the monosubstituted derivative.

The next step was to determine more closely at what stage of the 20 % acetone in hexane fraction the solasodine was eluting, and whether it was eluting completely in the 500 ml of solvent used. This was done by collecting 10 ml fractions off the silica gel column from when the 20 % acetone in hexane was first added, to when the first pure acetone fraction eluted. This totalled 60 fractions. During the

passage of the acetone front down the column, heating occurred. The "hot" fractions that subsequently eluted (fractions nine to 11) indicated the start of the acetone-containing fractions. In addition, considerable yellow colouration eluted just after these fractions. Similarly, more yellow-coloured compounds eluted at the start of the pure acetone fractions, thus marking fraction 60. All the fractions were then dried down in a vacuum centrifuge, redissolved in 300 μl 20 % acetone in hexane and 40 μl of each spotted onto TLC plates. The results are shown in Figure 21.

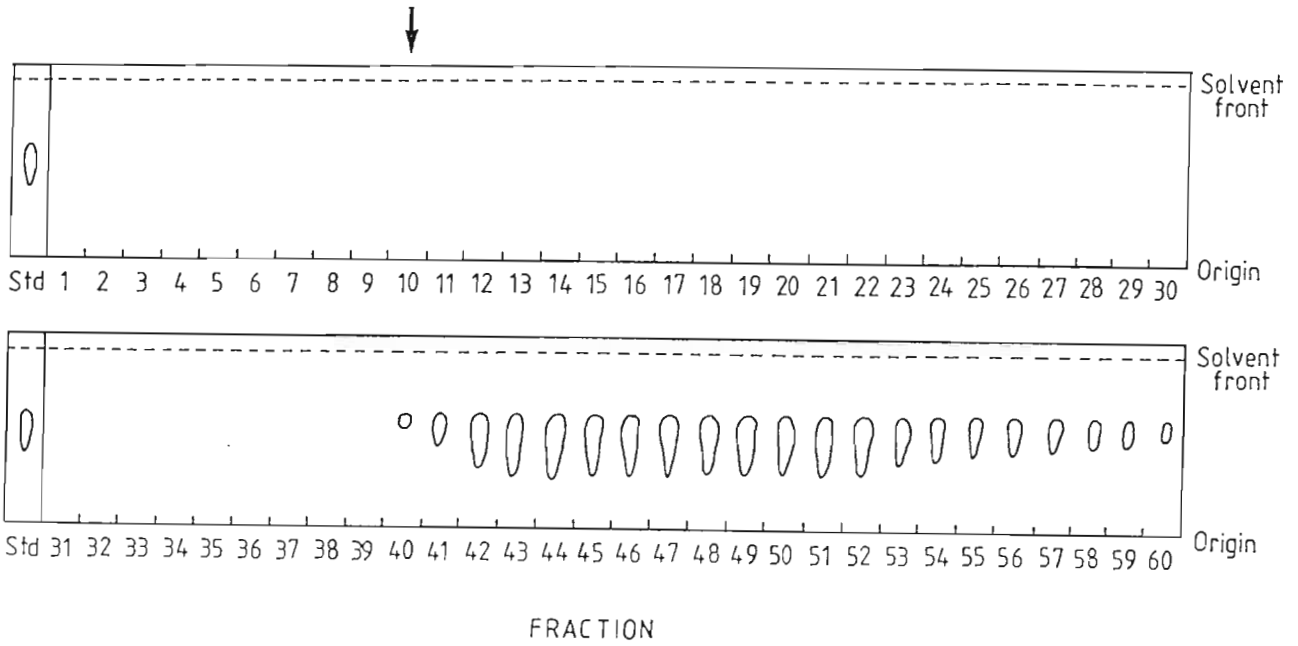

Figure 21. TLC results obtained following elution of solasodine from a silica gel column in 500 ml of 20 % acetone in hexane and collection of 10 ml aliquots. Solasodine was visualized with cobalt thiocyanate. The arrow indicates the start of the acetone-containing fractions.

Solasodine only commenced eluting in fraction 40 and although the amount eluting per fraction decreased after fraction 53, a considerable amount was still eluting in fraction 60. Rather than further increasing the volume of the eluent, to elute all the solasodine, the effect of increasing the percentage of acetone to 30 % was tested. This had a marked effect on the elution of solasodine (Fig. 22). Now, solasodine started to elute in fraction 21 and very little was still eluting in fraction 60. This procedure was therefore adopted for all subsequent trials.

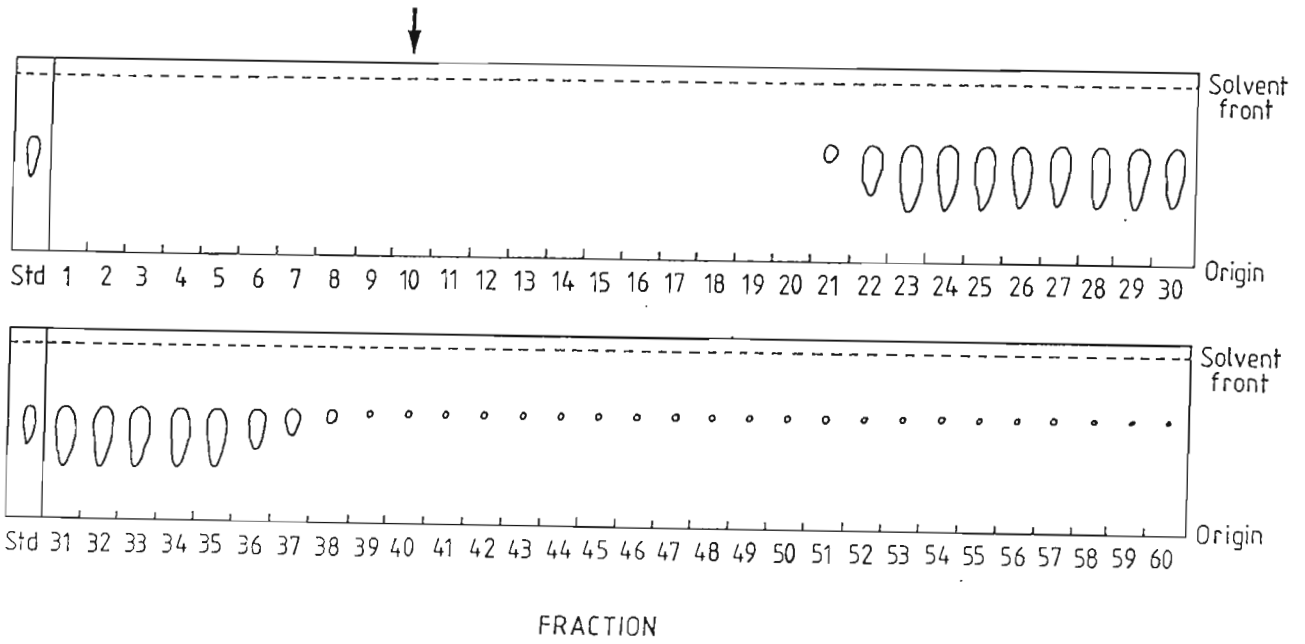

Figure 22. TLC results obtained following elution of solasodine from a silica gel column in 500 ml 30 % acetone in hexane and collection of 10 ml aliquots. Solasodine was visualized with cobalt thiocyanate. The arrow indicates the start of the acetone-containing fractions.

The remaining volumes of the solasodine-containing fractions were pooled, benzoylated and analyzed by HPLC. The results confirmed that more solasodine was recovered from the column when 30 % acetone in hexane was used, rather than 20 %, peak area being far less in the latter case.

One problem associated with the addition of 30 % acetone in hexane to the column, was that, together with the column heating, bubbles were released from the column, which severely disturbed the sample bed. It was therefore tested to see if a more gradual addition of the acetone would improve matters. This was done by adding 25 ml volumes of 5, 10, 15 and 20 % acetone in hexane, prior to the 30 % acetone in hexane. The release of bubbles was greatly reduced by these means, the column simply compressing slightly at this stage. This step was therefore included in all subsequent trials.

3.3.3. Further purification:

Although peak resolution had now improved, it was felt that further purification was still necessary and so a post-hydrolysis ethyl acetate wash was included in the extraction procedure. This was found to remove some yellow colouration from the extract. The pH of the sample was then increased to between eight and nine by the addition of 25 % ammonium hydroxide, the solasodine extracted with chloroform and passed through a silica gel column prior to benzoylation. Subsequent HPLC analysis of the sample indicated that this additional step resulted in the purification that had been sought (Fig. 23).

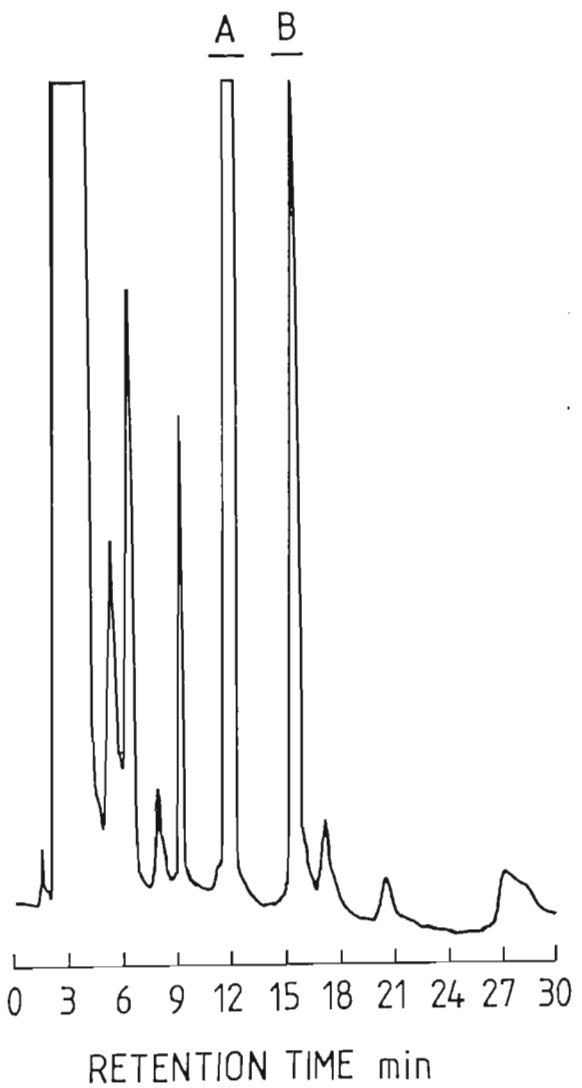

Figure 23. HPLC trace obtained following extraction of 5 g of freeze-dried *S. mauritianum* berry material and purification by silica gel column chromatography and a low pH ethyl acetate wash. A indicates the retention time of dibenzoylated solasodine and B, that of the monosubstituted derivative.

This was retested three times and each time large peaks co-eluting with authentic benzoylated solasodine were obtained, with minimum interference from other peaks. The acidic ethyl acetate wash was therefore included in the extraction and purification protocol. Use of this wash and exclusion of the silica gel column chromatographic purification step, yielded unsatisfactory traces. Presumably,

therefore, the two techniques remove different contaminants. Subsequently, both were included in all trials.

3.3.4. Product recovery:

A problem envisaged with the use of the fairly lengthy extraction and purification procedure, was that there would be considerable loss of solasodine during this process. It was deemed to be of interest and importance to have an indication of the magnitude of this loss. If attempting to compare solasodine contents of different *Solanum* species, extracted by different researchers, it is essential that this is taken into account. In order to test the level of losses, two samples of 3 mg of authentic solasodine were run through the entire extraction and purification procedure, from the hydrolysis stage onwards. Solasodine could not be added prior to this, since the chloroform partitioning would remove the aglycone. Following the silica gel chromatographic step, the samples were benzoylated and subjected to HPLC. The results were rather alarming, in that an average of only 29.9 ± 3.3 % of the original 3 mg of solasodine appeared to be recoverable. The entire procedure was repeated and a recovery of 35.8 % was obtained.

The question then asked was where the losses of solasodine were occurring. To test this, three samples of 3 mg of solasodine were again run through the extraction and purification steps and each of the fractions that were usually discarded along the way, were collected. They were dried down, benzoylated and tested by HPLC for the presence of solasodine. A considerable amount (4.7 ± 1.2 %) of solasodine was found to be in the acidic ethyl acetate wash. This was considered to be a

necessary loss, since inclusion of this step was essential for satisfactory HPLC detection of solasodine in plant extracts. The aqueous fraction from which solasodine was extracted prior to silica gel chromatography, contained only a trace of solasodine (0.233 %). Addition of these losses still resulted in a very large unaccountable loss. Possible losses on the silica gel column were therefore examined.

Three 3 mg samples of authentic solasodine were dissolved in chloroform and then dried onto 1 g of silica gel. The samples were then subjected to silica gel flash chromatography, as described. The hexane, 30 % acetone in hexane and pure acetone fractions were collected and dried down separately, prior to benzoylation, in preparation for HPLC analysis. In no instance was there even a trace of solasodine in the hexane fractions, whereas in the 100 % acetone fractions, a small amount (0.95 %) was recoverable. The 30 % acetone in hexane fractions, in which the solasodine should have eluted, yielded an average of 70.7 $\pm$ 3.9 %. This meant that approximately 30 % of the solasodine was being lost during the silica gel column chromatography, or in transfers subsequent to this step. As in the case of the acidic ethyl acetate wash, however, this step was considered to be essential for the determination of solasodine by the HPLC technique previously developed.

Overall, therefore, about 30 % of the solasodine was lost on the silica gel column and 10 % in fractions discarded during extraction and purification. Since the overall recovery was never found to be greater than 35 %, however, a loss of at least 25 % was still unaccounted for. Presumably this is attributable to solasodine breakdown at some point during extraction and purification, and losses during

sample transfers, despite the fact that all glassware was rinsed three times at each transfer. LANCASTER and MANN (1975) indicated that solasodine is readily adsorbed onto surfaces such as slightly etched glassware and recommended that all glassware be rinsed in an acetone solution of 0.5 % cresol and then oven-dried. This could help reduce losses, but it is very inconvenient to do this when a lengthy procedure, involving much glassware, is being followed.

The use of solid-phase extraction techniques (VOGEL, JATISATIENR and BAUER, 1990), in which the number of purification steps are reduced, would also help to reduce such sample losses. JONKER, KOOPS and HOOGENDOORN (1992) tested the recovery of steroidal glycoalkaloids from a wild *Solanum* species, *S. vernei* Bitter & Wittm., by spiking a tuber homogenate with purified steroidal glycoalkaloids. The only means of purification was solid-phase extraction. They found recovery for various glycoalkaloids to range from 96 to 101 %, an exceptionally good recovery. In contrast, FRIEDMAN and LEVIN (1992) obtained an overall recovery of only 70 % when using solid-phase extraction.

CRABBE and FRYER (1982), in their evaluation of a colorimetric solasodine determination technique, indicated that some researchers use repeated analysis of plant material to determine overall reproducibility. This will identify random, but not systematic errors. The approach used in the present study, in which spiking with pure solasodine was used to determine percentage recovery, is an accurate method of ascertaining losses. The important fact when using the developed extraction and purification techniques, was that the percentage recovery was consistent. This meant that the amount of solasodine obtained from different

samples, extracted and purified in the same way, could be compared, which was what was required in the present study. It is interesting to note how rarely the percentage recovery is presented in reported studies. Either, recoveries may be poor, as in the present case, and so not be reported, or else this is simply not tested. This means that the results of absolute levels of solasodine in different *Solanum* species, as determined by different researchers, cannot be compared, unless exactly the same procedures are followed. Perhaps the recent publication of papers such as one dealing purely with the quantification of polyamine losses during manipulation and assay procedures (DINNELLA, CRUES, D'ORAZI, ENCUENTRA, GAVALDA and SERAFINI-FRACASSINI, 1992), is an indication of an increasing awareness of the importance of determining percentage recoveries. If so, this is a very positive sign.

3.3.5. Determination of solasodine levels in different plant samples:

After an appropriate extraction and purification protocol had been developed and evaluated, a range of different plant samples were extracted, to test the applicability of the procedures. Ultimately, the aim was to extract solasodine from tissue cultures of *Solanum mauritianum*. Also, for interest and for later comparison with results from the tissue culture studies, the solasodine yield of different plant parts was determined. An entire plant (1 m high) of *S. mauritianum* was uprooted, cut up and the roots, stems and leaves freeze-dried. A leaf sample from a more mature plant was also collected and treated in the same way. The roots and stems were ground in a Wiley mill, while the leaves were crushed using a pestle and mortar. In addition, berries of *S. mauritianum* and another *Solanum* species, *S. aculeastrum*

Dun., were freeze-dried and ground. The latter species is an indigenous weed in South Africa (WELLS, BALSINHAS, JOFFE, ENGELBRECHT, HARDING and STIRTON, 1986), which has large fruit, up to 5 cm in diameter (GBILE, 1979). In all cases, the described procedures for extraction and purification were followed, prior to benzoylation of the samples and injection into the HPLC. Good, clear traces were obtained in all cases and the levels of solasodine were then calculated from a standard curve. The levels of solasodine obtained, uncorrected for any losses, are given in Table 7.

Table 7. Amount of solasodine extracted from various *Solanum* samples.

Sample	Solasodine $\mu g\ g^{-1}DW$
S. mauritianum roots	30
S. mauritianum stems	48
S. mauritianum leaves	21
Mature *S. mauritianum* leaves	10
S. mauritianum berries	184
S. aculeastrum berries	164

In the young *S. mauritianum* plant, most solasodine was extracted from the stem material, then roots and least from the leaves. This contrasts with the situation in *S. laciniatum* Ait. in which, although all parts of the young plant yielded some solasodine, immature and mature leaves were found to contain high concentrations of glycoalkaloids, whereas roots and stems had much less (LANCASTER and MANN, 1975). The amounts of the different types of glycoalkaloids in the leaves and roots of *S. laciniatum* also varied and it was suggested that either glycoalkaloid

synthesis is independent in different plant parts, or else a differential export of glycoalkaloids occurs, from the leaf to the root (LANCASTER, MANN and BLYTH, 1977). Glycoalkaloid composition was not examined in the present study, only solasodine yield.

It was thought that perhaps, as the *S. mauritianum* seedling develops and starts to produce berries, the levels of solasodine may become more concentrated in certain parts of the plant. This is suggested by the fact that the amount of solasodine obtained from the green berries of mature *S. mauritianum* plants was far greater than that from any part of the young plant. In addition, the solasodine yield from the mature leaves, was far lower than that from the leaves of the immature plant. The level of solasodine extracted from the berries of the other species that was examined, *S. aculeastrum* Dun., was slightly lower than that of *S. mauritianum*, but was of the same order (Table 7). The amount of solasodine extracted from *S. mauritianum* berries was the average of four extractions conducted at different times. The overall variation was less than 10 % of the mean. Therefore, subsequently, in the tissue culture trials, in the interests of saving both time and money, only a single extraction was conducted per treatment. In addition, less plant material is then required for extraction purposes, which is important, considering that one would generally expect levels of secondary products to be lower *in vitro* than *in vivo*.

The amount of solasodine extracted from *S. mauritianum* in the present study can be calculated to 0.018 % on a dry weight basis. This is considerably lower than the 0.16 % recorded by CAMPBELL and VAN STADEN (1990) and almost 100

times less than the 1.7 % level reported by SCHREIBER (1963 in MANN, 1978 survey). Such a large discrepancy is difficult to explain. One reason could be that the plants from which berries were collected in the present study were not growing under optimal conditions, as a crop would. Rather, these weeds were usually growing in harsh, hot, dry situations. Perhaps such conditions are not conducive to solasodine production by the plant, the trigger for which is unknown. Geographical differences are also known to affect solasodine production greatly (MILLER and DAVIES, 1978; BHARATI, PANDA and CHATTERJEE, 1986). A large proportion of the discrepancy in reported solasodine levels may, however, not be a real difference at all. Rather, it may result from the use of different extraction and purification techniques, which, as discussed previously, can result in very different sample recoveries. In surveys conducted of solasodine yields, no indication is given whether the values are corrected for any losses or not. In addition, the means by which solasodine is analyzed will affect the result obtained. The more crude the alkaloid fraction can be for successful analysis, the less the purification that is required, and thus, the less the losses will probably be, which could lead to artificially high yield values. If solasodine is to be exploited, it is the level of pure solasodine that can be obtained, that is important. More comparable are the results obtained in studies such as that of BRADLEY, COLLINS, EASTWOOD, IRVINE, SWAN and SYMON (1979), who, in a single study, examined 84 Australian *Solanum* species for their steroidal alkaloid content. They found that 20 of these species contained less than 0.1 % alkaloid in any plant part, 34 contained alkaloids in the fruit only and the remaining 30 were found to have alkaloids in leaf, stem and fruit samples. WEILER, KRÜGER and ZENK (1980) undertook a similar study of herbarium material of over 200 *Solanum* species.

Unfortunately, only leaf material was used. They found 61.8 % of the species examined, including *S. aculeastrum*, to contain between 0 and 0.1 % solasodine in the leaves, which is very low.

Overall, therefore, the yield of solasodine obtained from the green berries of *S. mauritianum* in the present study, appears to be very low, relative to other reports for this species, although this is comparable to the levels reported in many other *Solanum* species.

3.4. Conclusions

A suitable extraction and purification protocol was established to complement the use of the developed benzoylation and HPLC analysis techniques. Losses were calculated to be considerable, with only 30 to 35 % of the solasodine present being recovered. The loss was consistent. The techniques established, were used to extract solasodine from leaf, stem, root and berry samples. None of these samples presented any difficulties during the extraction and purification steps. In all cases, peaks of benzoylated solasodine were clearly detectable on the HPLC traces, with little interference from other compounds. It was therefore considered that the techniques should be easily applicable to tissue-cultured samples, such as callus.

The green berries of *S. mauritianum* were found to contain detectable levels of solasodine, although they were low relative to other reports. If, however, collection of berries was an integrated part of a weed control programme, as suggested by CAMPBELL and VAN STADEN (1990), utilization of the solasodine could still be

a viable proposition. Alternatively, the use of very controlled conditions, such as those that prevail in tissue culture systems, could stimulate increased production of solasodine. This aspect will be addressed in the following chapters.

CHAPTER 4

SECONDARY PRODUCT SYNTHESIS IN CULTURE

4.1. Introduction

Various aspects related to the synthesis of secondary products *in vitro* have previously been mentioned (Chapter 1.4). These include the advantages of the synthesis of secondary metabolites in culture over their extraction from intact plants; certain associated problems such as high costs and low productivity; and some of the successes that have been achieved in this field. In addition, factors that influence secondary product production in culture, such as medium composition, light, temperature and stress were discussed. In this chapter, certain guidelines that have been developed in the past, following observation of secondary product synthesis *in vitro*, will be mentioned, as well as the various culture types employed for this purpose. This will serve as a general introduction to the following three chapters, in which *Solanum mauritianum* cultures of various types will be described.

4.2. Source material

When selecting a species for the *in vitro* synthesis of a desired chemical product, one would naturally choose a high-yielding species, in the expectation that it would give rise to a high-yielding culture. Frequently, however, cultures do not reflect the biosynthetic capacity of the source plant (KURZ, 1986). It has been concluded

that there are differences between species in their ability to express genetic information when cultured. The genes that contain the genetic information for the enzymes required to catalyze the biosynthetic reactions appear to be dormant or repressed in certain species under conditions of culture (HEINSTEIN, 1986). The establishment of cultures from lower-yielding species has, in some cases, resulted in high-yielding cultures (KURZ and CONSTABEL, 1985). Much of this form of success may be related to differences in culture conditions. Seemingly minute differences in culture conditions can result in substantial differences in product quality and quantity (KURZ, 1986). Therefore, although *Solanum mauritianum* was found to yield relatively low levels of solasodine (Chapter 3.3.5), this does not exclude the possibility of it giving rise to high-yielding cultures.

4.3. Synthesis of secondary metabolites in differentiated and non-differentiated cultures

The concept of totipotency, which allows entire plants to be regenerated from single cells or callus, originally resulted in the expectation that because a cell had the potential to do all that the plant could, it would (GRESSEL, 1980). However, in reality, the synthesis of secondary products has often been found to be connected with cell differentiation, differentiation being associated with an increase in secondary metabolite production (COLLINGE, 1986; COLLIN, 1987; CONSTABEL, 1988; SCHMAUDER and DOEBEL, 1990). Some researchers (FOWLER, 1986) do not regard this as surprising, since in the intact plant, most secondary products are synthesized in cells that have undergone a terminal development to form part of a complex tissue, or, at the very least, in cells that

have ceased to grow and divide. The low productivity in non-differentiated cultures has led to increasing interest in cultures containing different organs, such as multiple shoot and root cultures. Despite these problems, however, economically-viable levels of secondary products have been produced in undifferentiated cell suspension cultures. A good example of this is the production of shikonin in suspension cultures of *Lithospermum erythrorhizon* Lieb. et Zucc., which was mentioned previously (Chapter 1.4). In *Hyoscyamus muticus* L. (KOUL, AHUJA and GREWAL, 1983) and *Papaver somniferum* L. (SIAH and DORAN, 1991) cultures, secondary product synthesis has been found to be independent of morphogenesis. Therefore, culture systems involving differentiated or non-differentiated cells both appear to have potential in the synthesis of secondary products and are therefore worthwhile avenues to explore.

4.4. Line selection for secondary product synthesis

Variation arises as soon as tissues are excised from a parent plant and placed in culture. It is common to all cultures as well as within a culture. The original explants are usually heterogeneous, consisting of different cell types that, on proliferation, lead to a complex cell population consisting of cells with differing growth rates, ploidy levels and physiology (HOLDEN, HOLDEN and YEOMAN, 1988). This results in differences in levels of secondary products synthesized in different cell lines. In cases where several compounds of interest are produced in a single culture, variation will affect the entire pattern of compounds and result in lines that produce none, few, or many compounds (KURZ, 1986; KURZ and CONSTABEL, 1985).

A problem arises in that the relationship between growth rate and secondary metabolite synthesis is primarily a reverse one. Therefore, during initial culture procedures, an unconscious selection may occur for cells showing rapid proliferation, but these may prove to have a low capacity for secondary product synthesis (SCHMAUDER and DOEBEL, 1990). In order for the synthesis of a secondary metabolite *in vitro* to be viable, the selection of a line with a high yield is advantageous. This is most easily applicable to cultures that accumulate coloured products, such as shikonin, since then high-yielding lines can be selected visually, without the need for elaborate analyses (RHODES, ROBINS, HAMILL and PARR, 1986). The development of radioimmunoassays and enzyme immunoassays, which are very sensitive analytical techniques, has helped greatly in the selection of cell clones with high yields of non-coloured, assayable constituents (ROBERTS, 1988).

It is recommended that once a high-yielding clone has been selected, it should be manipulated to produce the highest yield possible and a high-yielding, stable line should be developed (FOWLER, 1986). However, DEUS-NEUMANN and ZENK (1984) found that in *Catharanthus roseus* (L.) G. Don., selection of high indole alkaloid yielding cell lines favoured an inherent instability. For this reason, selected lines should be subjected to continuous screening throughout all subcultures (SCHMAUDER and DOEBEL, 1990). This instability is far greater than that observed in high-yielding microbial cultures (RHODES, ROBINS, HAMILL and PARR, 1986). The magnitude of such change in established cell lines is apparently species dependent (CURTIS, HASEGAWA and EMERY, 1991). In order to reduce variation in product formation by established cell lines, the protocol for

subculturing should be strictly adhered to, since any deviation may significantly alter the level of production (KURZ, 1986). If plant cell cultures are to be used on an industrial scale, large variations between batches must be reduced as much as possible, for the process to be viable.

4.5. Culture types used for secondary product synthesis

A wide variety of culture systems can be used for the synthesis of secondary products and all have some advantages and certain disadvantages. The basic culture types are:

i) Differentiated cultures

ii) Callus cultures

iii) Suspension cultures

4.5.1. Differentiated cultures:

The frequently-observed phenomenon that differentiated cells produce more secondary products than non-differentiated cells, has led to the development of several culture systems for secondary product synthesis, which contain morphologically differentiated cells. Good yields of secondary products have been obtained in some instances, but such cultures frequently present mechanical difficulties in bioreactors.

4.5.1.1. Leaf cultures:

These are unusual cultures which are not frequently reported. In *Catharanthus roseus* (L.) G. Don., leaves were initiated from callus cultures. These leaves were then cultured in the absence of any root tissue or dedifferentiated callus and maintained as a shake culture. An inoculum of 2.5 g of leaf material resulted in 29 g (fresh weight) after 35 days of growth. The leaves were then extracted and preliminary studies showed the presence of vindoline and a complex variety of other alkaloids. Unfortunately, no indications of yield were given (KRUEGER, CAREW, LUI and STABA, 1982).

4.5.1.2. Shoot cultures:

Shoot cultures may consist of only shoots (ENDO, GOODBODY and MISAWA, 1987; ROJA, BENJAMIN, HEBLE, PATANKAR and SIPAHIMALANI, 1990) or, more usually, of callus that produces multiple shoots. Examples of instances in which multiple shoot cultures have been used include the production of indole alkaloids in *Catharanthus roseus* (HIRATA, YAMANAKA, KURANO, MIYAMOTO and MIURA, 1987), triterpenoid saponins in *Gypsophila* species (PAUTHE-DAYDE, ROCHD and HENRY, 1990) and tropane alkaloids in *Duboisia leichhardtii* F.Muell. (YAMADA and ENDO, 1984). Variable yields have been reported. In some cases, the desired secondary products have been produced in multiple shoot cultures at levels equal to or greater than those of the intact plant (HIRATA, YAMANAKA, KURANO, MIYAMOTO and MIURA, 1987; ROJA, BENJAMIN, HEBLE, PATANKAR and SIPAHIMALANI, 1990).

More usually, however, yields are lower than those of the parent plant, or else none of the desired product is detected at all (YAMADA and ENDO, 1984; KITAMURA, MIURA and SUGII, 1985). In a few cases, the secondary product content of multiple shoot cultures has been found to be equivalent to that of callus of the same species (PAUTHE-DAYDE, ROCHD and HENRY, 1990), thus refuting the general trend that differentiated tissues produce higher levels of secondary products than undifferentiated tissues.

4.5.1.3. Root and hairy root cultures:

In general, the use of root cultures seems to have been more successful in the *in vitro* production of secondary products than shoot or leaf cultures. In *Duboisia myoporoides* R. Br. callus and shoots differentiated from callus were found to contain no tropane alkaloids, whereas they were detectable in roots differentiated from the callus (KITAMURA, MIURA and SUGII, 1985). The use of root cultures has been recommended in those situations in which roots constitute a main site of synthesis of the desired product (STAFFORD, 1991), as is the case for alkaloids. The roots are either initiated from callus by manipulation of the hormone complement, as for example in *Duboisia* species (KITAMURA, SUGIMOTO, SAMEJIMA, HAYASHIDA and MIURA, 1991), or else root tips are excised from *in vitro* grown seedlings and grown as liquid shake cultures, as for example in *Cephaelis ipecacuanha* Rich. (JHA, SAHU, SEN, JHA and MAHATO, 1991). A problem frequently encountered with root cultures is that they cannot be grown indefinitely. Root cultures have primarily been examined as potential producers of various types of alkaloid, such as pyrrolidizine alkaloids in *Senecio* species

(TOPPEL, WITTE, RIEBESEHL, VAN BORSTEL and HARTMANN, 1987), tropane alkaloids in *Hyoscyamus* (HASHIMOTO, YUKIMUNE and YAMADA, 1986) and *Duboisia* (ENDO and YAMADA, 1985), and indole alkaloids in *Catharanthus roseus* (ENDO, GOODBODY and MISAWA, 1987). Success has frequently been obtained with the synthesis of these compounds in root cultures and in some cases, secondary metabolite production has been comparable to (TOPPEL, WITTE, RIEBESEHL, VAN BORSTEL and HARTMANN, 1987; KITAMURA, SUGIMOTO, SAMEJIMA, HAYASHIDA and MIURA, 1991) or greater (JHA, SAHU, SEN, JHA and MAHATO, 1991) than that of the source plant. One difficulty with this type of comparison, however, is that the secondary product content of the young, cultured roots is compared with that of established plants, whose roots may be several years old. A major problem associated with root cultures is that, even if the yield of secondary products is very high, potential commercial exploitation is hampered by the fact that the cultures tend to grow very slowly. A more promising development has been the use of root cultures in which the roots have been transformed by *Agrobacterium rhizogenes*, the bacterium which causes hairy root disease (COLLINGE, 1986).

Infectious hairy root disease has a wide host range amongst dicotyledonous species in nature. A typical symptom of the disease is the formation of a mass of adventitious roots on the stem of the host plant (DE CLEENE and DE LEY, 1981). The underlying mechanism is the transfer of several genes to the plant genome, mediated by the bacterium. These genes, the root loci or *rol* genes, are located on a piece of DNA, the T(ransfer)-DNA, that is part of a large plasmid, the root-inducing or Ri plasmid (VAN WORDRAGEN, OUWERKERK and DONS, 1992).

This DNA codes for auxin synthesis and other rhizogenic functions (PARR and HAMILL, 1987). The expression of Ri-T-DNA in plant cells results in the production of transformed, hairy roots at the site of infection (OTTAVIANI, SCHEL and HÄNISCH TEN CATE, 1990). In addition, the T-DNA directs the infected plant to synthesize certain strain-specific metabolites, known as opines. These may then be used by the bacterium as the sole source of carbon and nitrogen (GELVIN, 1990; ISOGAI, FUKUCHI, HAYASHI, KAMADA, HARADA and SUZUKI, 1990). The presence of these compounds provides evidence for the transformation.

A potentially useful effect of this long-known, soil-borne bacterium has now been recognized (STAFFORD, 1991), namely the deliberate infection of susceptible plant cells with *Agrobacterium rhizogenes* to produce roots which can be cultured. It was TEPFER and TEMPÉ (1981) who first demonstrated that roots transformed by *A. rhizogenes* could be cultured aseptically *in vitro*. These roots typically exhibit diminished geotropism (OTTAVIANI, SCHEL and HÄNISCH TEN CATE, 1990), a high degree of lateral branching and, once produced, may be cultured in the absence of externally-supplied hormones (SHARP and DORAN, 1990; SAITOU, KAMADA and HARADA, 1991). They have a profusion of root hairs and a very high growth rate, exceeding that of nearly all untransformed root cultures and most cell suspension cultures (HAMILL, PARR, RHODES, ROBINS and WALTON, 1987). An important feature of hairy root cultures is that they have been shown to be capable of permanent growth and are chromosomally stable (ONDŘEJ and BÍSKOVÁ, 1986).

It was in the mid 1980's that researchers first demonstrated the production of secondary metabolites by hairy root cultures (FLORES and FILNER, 1985; HAMILL, PARR, ROBINS and RHODES, 1986; MANO, NABESHIMA, MATSUI and OHKAWA, 1986). Since then, an increasing number of reports have appeared of species in which hairy root cultures have produced levels of secondary metabolites, usually alkaloids, equal to or greater than intact plants (KAMADA, OKAMURA, SATAKE, HARADA and SHIMOMURA, 1986; KNOPP, STRAUSS and WEHRLI, 1988; GRÄNICHER, CHRISTEN and KAPÉTANIDIS, 1992).

The rapid growth and stable production of high levels of secondary products in hairy root cultures, has led to much interest in the possible commercial exploitation of such systems. The successful growth of hairy root cultures in bioreactors has already been achieved (RODRÍGUEZ-MENDIOLA, STAFFORD, CRESSWELL and ARIAS-CASTRO, 1991; WHITNEY, 1992). Overall, therefore, of the different types of differentiated cultures, it is the hairy root cultures that appear to have the most potential with respect to secondary product synthesis *in vitro*. Since the technique appears to be especially applicable to alkaloid production, and solasodine is a steroidal alkaloid, this seemed a viable potential avenue to explore in the current study.

4.5.2. Callus cultures:

A callus is a wound response from a plant (SMITH, 1986). In the present context, callus is initiated when an explant is removed from an intact plant, sterilized and transferred to a medium supplemented with plant growth regulators. Subsequently,

cell proliferation occurs and callus forms (ALLAN, 1991). Individual cells in a callus can vary in size, shape, pigmentation and appearance. Most have a large central nucleus and a vacuole to one side (SMITH, 1986). Callus can be removed from the original explant and maintained *in vitro* by routine subculture (ALLAN, 1991). The cells are totipotent and, as such, should have the potential to synthesize any of the compounds normally associated with the intact plant. In secondary product research, callus cultures themselves may be examined as potential sources of secondary metabolites, or else, they may be used as a stepping-stone in the establishment of suspension cultures (Chapter 4.5.3). The production of a wide range of secondary products has been attempted in callus cultures, including saponins (FURUYA, YOSHIKAWA, ISHII and KAJII, 1983; PAUTHE-DAYDE, ROCHD and HENRY, 1990), various types of alkaloids (DELFEL, 1980; VERPOORTE, MULDER-KRIEGER, WIJNSMA, VERZIJL and SVENDSEN, 1984; MORRIS, 1986a) and shikonin (HARA, MORIMOTO and FUJITA, 1987).

In many instances, the level of secondary products has been found to be very low under the culture conditions employed (SCRAGG and ALLAN, 1986; COLOMBO and TOME, 1991; BAUMERT, GRÖGER, KUZOVKINA and REISCH, 1992). However, the synthesis of significant amounts of secondary metabolites in callus cultures has also been reported in several species. This includes the production of histamine in *Helianthus annuus* L. (KAMAL and KHANNA, 1979), catechins in *Fagopyrum esculentum* Moench. (MOUMOU, VASSEUR, TROTIN and DUBOIS, 1992) and purine alkaloids in cocoa (GURNEY, EVANS and ROBINSON, 1992) cultures. In the latter case, callus cultures were found to be more productive and

less variable than suspension cultures of the same species. Therefore, callus cultures do appear to have the potential to produce considerable amounts of secondary metabolites under the correct conditions, and are therefore worth investigating.

4.5.3. Suspension cultures:

At present, suspension culture seems to be the culture type most widely used for the synthesis of secondary products *in vitro* and as a result, various modifications of the basic suspension culture have been developed. A summary of the research papers concerning secondary metabolite synthesis in suspension cultures would have thousands of entries. Suspensions are initiated by placing small pieces of friable callus into a liquid medium and shaking the culture (ALLAN, 1991), for example on an orbital shaker at 50 to 200 rpm. Friable callus can be visually selected and subcultured from compact, hard cultures, or it can be achieved by manipulating medium components, such as the type and concentration of auxin and cytokinin. If too little callus is used as inoculum, the culture may not grow. Generally, 2 to 3 g of callus is inoculated into 100 ml of medium (SMITH, 1986). If the culture conditions are suitable, the cells will increase in size and ultimately divide (ALLAN, 1991).

The amount of cell material will increase for a limited period only. If, at this stage, the culture is diluted back by subculture into fresh medium, the culture should commence a similar pattern of growth and yield. At the time of the first subculture, the suspension will contain residual clumps of inoculum as well as more

finely-dispersed aggregates and free cells. The larger clumps should be excluded during subculture by filtration or by allowing the clumps to settle out and be left behind (STREET, 1977). Suspension cultures require more frequent subculture than callus cultures, namely at one to three week intervals, depending on the amount of inoculum required and the vigour of the culture (SMITH, 1986). Although this means that suspensions must be treated more intensively, it also means that biomass can be produced more quickly. This growth rate is, however, still much slower than the five hours to seven days of fungal and bacterial cultures (HEINSTEIN, 1986). Another advantage of suspension cultures over callus cultures is that they can relatively easily be scaled-up and grown in a bioreactor, which is essential if any system is to become commercially viable.

The growth curve of plant cell suspensions typically consists of five segments, namely the lag, exponential, linear, progressive decelerating and stationary phases (DODDS and ROBERTS, 1985; SMITH, 1986; Figure 24).

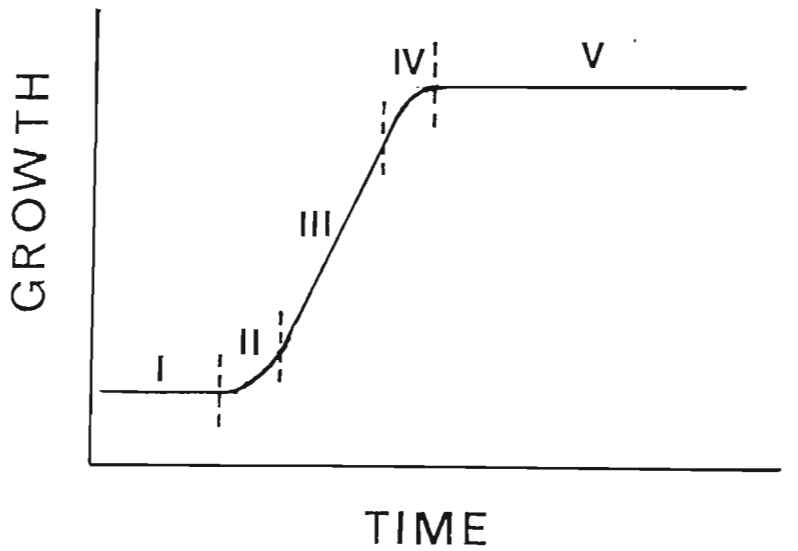

Figure 24. Typical growth curve of a suspension culture, showing the lag (I), exponential (II), linear (III), progressive decelerating (IV) and stationary (V) phases.

It is best to subculture cells at a defined stage of growth, usually prior to reaching the stationary phase (ALLAN, 1991).

Reports of cell suspension cultures giving yields of secondary products approaching or exceeding those of the intact plant, began to appear in the 1970's. Prior to this, the yields were usually lower by several orders of magnitude (DOUGALL, 1981). In many cell cultures, the media required for cell growth and secondary product synthesis may be very different, resulting in the use of a two-stage culture system. During the first stage, conditions are optimized for the growth of the cells, while during the second, conditions are changed to stimulate maximum secondary metabolite production (BECKER, 1987; FUJITA and TABATA, 1987). This is the system that is used in the commercial production of shikonin in Japan (Chapter 1.4). Economically, however, a single stage process, not requiring any alteration of culture conditions, would be more viable.

Most cells store secondary metabolites in vacuoles (HEINSTEIN, 1986), making it necessary to extract the products from the cell cultures, rather than the medium, thus destroying the cells. If the product could be recovered from the medium, the biomass would not need to be destroyed and the desired secondary product could be recovered on a continuous or semi-continuous basis, potentially in a partially purified form (ROBINS, PARR and RHODES, 1988). This latter approach would lend itself to use in a bioreactor. There have been several attempts to permeabilize cells in culture by the addition of certain chemicals, thus forcing the cells to release their products. The most commonly-used chemical is dimethyl sulfoxide (DMSO) (CURTIN, 1983). This has been successful in some cases, for example *Coleus*

blumei Benth. (PARK and MARTINEZ, 1992). However, more frequently, the DMSO has had a deleterious effect on the cell membranes, for example in *Cinchona ledgeriana* Moens. (PARR, ROBINS and RHODES, 1984).

An alternative approach is to immobilize the cells, since, for unknown reasons, the membrane permeability problem encountered in suspension cultures is less marked in immobilized cells of certain species (HEINSTEIN, 1986). The method most commonly used for immobilization is gel entrapment with calcium alginate. A mixture of sodium alginate and plant cells is extruded dropwise into a calcium chloride solution, where bead hardening occurs (BUITELAAR and TRAMPER, 1992). The beads, which then contain the cells, are shaken, as in a normal suspension culture. The cells proliferate within the beads and remain alive and capable of producing secondary metabolites, in the immobilized state (BRODELIUS and MOSBACH, 1982). Other forms of immobilization include adsorption to support materials such as fibreglass, metals, plastics and ceramics (BUITELAAR and TRAMPER, 1992) and immobilization in polyurethane foam cubes (MAVITUNA and PARK, 1985; ISHIDA, 1988).

The use of immobilized cells also has certain other advantages over free suspension cultures: The cells are protected against shear forces and consequently, a simpler bioreactor design may be used; a higher cell density is possible (BRODELIUS, 1985); and cell washout is reduced (SCOTT, 1987). In instances where immobilized cell cultures are found to be efficient producers of secondary metabolites, a classical suspension is used for the first growth stage and immobilized cells for the second, productive stage of culture.

Secondary products have been produced by immobilized cells of many different species, including *Coffea arabica* L. (FURUYA, KOGE and ORIHARA, 1990), *Nicotiana tabacum* L. (HAIGH and LINDEN, 1989) and *Dioscorea deltoidea* Wall. (ISHIDA, 1988). Frequently, the product yield has been lower than that of the corresponding free cell suspension culture. However, immobilized cells may keep on producing the secondary product for a longer period, for example six months in *Papaver somniferum* L. (SCRAGG, 1991), making overall productivity greater than that of free cells (BUITELAAR and TRAMPER, 1992).

One field in which immobilization of suspended cells is proving useful is in the *in vitro* production of secondary metabolites through biotransformation. In this process, precursors of the desired product are added to the medium in an attempt to result in their incorporation into and hence enhanced yields of the secondary product. Thus, the enzymatic potential of the cultured plant cells is used for bioconversion purposes. The advantages are that the enzymes catalyze reactions stereospecifically and can perform regiospecific modifications that are not easily carried out by chemical synthesis or microorganisms (PRAS, 1992).

Biotransformation has been successfully conducted with suspension cultures (SASSE, WITTE and BERLIN, 1987; INOMATA, YOKOYAMA, SETO and YANAGI, 1991), root cultures (TOPPEL, WITTE, RIEBESEHL, VAN BORSTEL and HARTMANN, 1987; KITAMURA, TAURA, KAJIYA and MIURA, 1992) and hairy root cultures (KAWAGUCHI, HIROTANI, YOSHIKAWA and FURUYA, 1990; ROBINS, PARR, PAYNE, WALTON and RHODES, 1990), in addition to immobilized cells. Whatever the culture type used, however, it is firstly essential

that there is a basic understanding of the pathway of synthesis of the desired secondary product, so that the correct precursors can be added to the medium. This poses a problem with potential biotransformation to form solasodine, since its mode of biosynthesis is unclear (Chapter 1.3.2.1).

4.6. Conclusions

There are certain guidelines that can direct a researcher to develop a potentially successful *in vitro* system for secondary product synthesis, such as the increased production of secondary products by differentiated cells over non-differentiated cells. However, for every such general principle, there are many exceptions. Similarly, there are many different culture systems that can be used, and all have been found to be successful in some species. Certain types, however, such as hairy root and suspension cultures, exhibit positive results more consistently and are therefore more likely to yield success. In the following three chapters, initiation of several of the different culture types mentioned will be attempted, with a view to producing solasodine in *Solanum mauritianum* cultures.

CHAPTER 5

CALLUS CULTURES OF *SOLANUM MAURITIANUM*

5.1. Introduction

Callus cultures have been initiated from numerous *Solanum* species in the past. These have included edible species, such as potato and eggplant, as well as wild species. The latter have been cultured primarily with a view to producing some type of alkaloid *in vitro* (ALVAREZ, NIGRA and GIULIETTI, 1993). In this case, the callus itself may be extracted for the secondary products, or else, suspension cultures may be initiated from it, for the same purpose. Alternatively, callus is initiated for micropropagation and breeding purposes, to increase the alkaloid content of the intact plants (BANERJEE, SCHWEMMIN and SCHWEMMIN, 1985). A summary of some wild *Solanum* species in which callus cultures have been successfully initiated, is given in Table 8. The explant type used and an indication of the medium composition and hormone complement are also provided.

Table 8. Wild *Solanum* species from which callus cultures have been initiated successfully.

Species	Explant	Medium	Hormones	Reference
S.xanthocarpum Schrad. et Wendl.	Stem	M & S	Coconut milk 2,4-D Adenine	HEBLE, NARAYANA-SWAMI & CHADHA (1971)
S. aviculare Forst. *S. eleagni-folium* Cav. *S. nigrum* L. *S. khasianum* Clarke	Seedlings	Revised M & S (RT)	2,4-D	KHANNA, UDDIN, SHARMA, MANOT & RATHORE (1976)
S. laciniatum Ait.	Seedlings	M & S Yeast extract	2,4-D	HOSODA & YATAZAWA (1979)
S. khasianum Clarke	Seedlings	M&S B5	not given	KOKATE & RADWAN (1979)
S. nigrum L.	Green fruit	White's	Coconut milk 2,4-D	MANDAL & GADGIL (1979)
S. khasianum Clarke	Radicle, leaf, shoot apex, seedling	Revised M & S (RT)	IAA 2,4-D Kinetin	UDDIN & CHATURVED I (1979)
S. jasminoides Paxt.	Stem cuttings	Revised M & S (RT)	not given	JAIN & SAHOO (1981a)
S. verbascifolium L.	Seeds	Revised M & S (RT)	2,4-D	JAIN & SAHOO (1981b)

Species	Explant	Medium	Hormones	Reference
S. laciniatum Ait	Nodal segments with axillary buds, leaves	M & S salts, own vitamins	NOA BA	CONNER (1982)
S. nigrum L.	Leaves	M & S salts, Gamborg vitamins	IAA BA	BHATT, BHATT & SUSSEX (1983)
S. laciniatum Ait.	Leaves	M & S	2,4-D Kinetin	CHANDLER & DODDS (1983)
S. sarrachoides Sendt	Petiole, leaf, flower	M & S salts, own vitamins	NAA BA	BANERJEE, SCHWEMMIN & SCHWEMMIN (1985)
S. dulcamara L.	Hypocotyl Stem	M & S	2,4-D Kinetin	EMKE & EILERT (1986)
S. mammosum L.	not given	M & S	2,4-D Kinetin	INDRAYANTO & SUTARJADI (1986)
S. glaucophyllum Desf.	Seedlings	Revised M & S (RT)	2,4-D	JAIN & SAHOO (1986)
S. laciniatum Ait.	Leaves	M & S	NOA BA	CONNER (1987)
S. platanifolium Sims	Berries	M & S	2,4-D Kinetin	JAGGI, BHATNAGAR, QADRY & KAPOOR (1987)
S. viarum Dun. *S. sisymbrifolium* Lam.	Leaves	M & S	IAA Kinetin	MATTOS & CORDEIRO (1989)

Species	Explant	Medium	Hormones	Reference
S. eleagnifolium Cav.	Berries, Seedling hypocotyl cotyledon roots & leaves	Revised M & S (RT)	2,4-D	NIGRA, CASO & GIULIETTI (1987)
S. aviculare Forst.	Seedling roots	M & S	NOA BA	TSOULPHA & DORAN (1991)

Abbreviations:
Gamborg: GAMBORG, MILLER and OJIMA's (1968) medium; M & S: MURASHIGE and SKOOG's (1962) medium; RT: vitamins of KHANNA and STABA (1968); White: WHITE's (1963) medium.
BA: benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; IAA: indoleacetic acid; NAA: naphthaleneacetic acid; NOA: naphthoxyacetic acid.

None of the above authors indicated much difficulty in initiating callus from wild *Solanum* species. The explant chosen varied greatly, and included stems, leaves, seedlings, flowers and fruit. With few exceptions, the nutrient medium used was that of MURASHIGE and SKOOG (1962), either in the original form, or with various modifications. These modifications have usually arisen with respect to the vitamins. The use of the vitamin supplement of KHANNA and STABA (1968), known as RT, is frequently reported (details given in Chapter 6). A range of different hormones has been used. Usually, both auxins and cytokinins seem to be used, although sometimes no cytokinin appears to be necessary. The auxin most commonly included in the medium is 2,4-D, while BA and kinetin are used as cytokinins.

Solasodine was found to be present in callus cultures of certain of these *Solanum* species (Table 9), although usually in relatively small amounts, as is frequently the case with secondary metabolite synthesis in callus cultures (Chapter 4.5.2). These results were obtained by manipulation of a whole host of different culture conditions, including variation in medium makeup, hormonal complement of media, light and temperature.

Table 9. *Solanum* callus cultures in which the presence of solasodine has been detected.

Solanum species	Solasodine content	Reference
S. laciniatum Ait.	0.30 - 0.5 mg g^{-1}DW	HOSODA, ITO & YATAZAWA (1979)
	0.02 - 0.38 mg g^{-1}FW	CHANDLER & DODDS (1983)
	0.09 mg g^{-1}DW	CONNER (1987)
S. eleagnifolium Cav.	0.01 - 1.3 mg g^{-1}DW	ALVAREZ, NIGRA & GIULIETTI (1993)
S. nigrum L.	0.005 - 0.15 mg g^{-1}FW	BHATT, BHATT & SUSSEX (1983)
S. verbascifolium L.	0.023 - 0.054 mg g^{-1}DW	JAIN & SAHOO (1981b)
S. glaucophyllum Desf.	0.012 - 0.037 mg g^{-1}DW	JAIN & SAHOO (1986)

Overall, the levels of solasodine obtained generally appear to be in the low microgram range, when measured on a dry weight basis. It is interesting to note that in *S. laciniatum*, for which callus cultures have been established by several

different researchers, the solasodine levels vary considerably. This is not necessarily only as a result of different culture conditions and parent plants, but may also be affected by the different extraction, purification and determination techniques used, as discussed in Chapter 3. In some *Solanum* species, for example *S. mammosum* L. (INDRAYANTO and SUTARJADI, 1986), no solasodine was detectable in callus cultures with the specific conditions and techniques used.

With the knowledge gained from the above brief survey, it seemed that callus should be readily initiated from explants of *S. mauritianum* and that solasodine might be detected in this callus.

5.2. Materials and Methods

Leaf, stem and berry explants were initially tested as potential sources of callus-producing material. However, as will be shown in Chapter 7, leaves of *S. mauritianum* are tremendously difficult to sterilize efficiently. A similar problem was encountered with stem material. Therefore, subsequent callus initiation trials were conducted with berry explants only. Young, green berries were used, which, when halved, were green throughout. If the seeds already had a brown colour, the fruit were rejected. The way in which the explants were obtained is illustrated in Figure 25. They consisted of immature seeds, placental and inner fruit wall tissue. Each berry yielded four such explants.

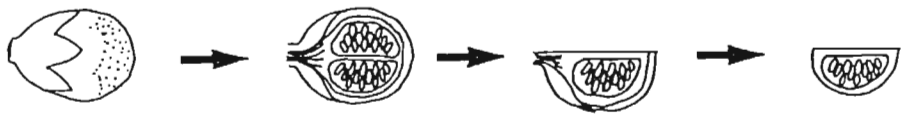

Figure 25. Excision of explants from green *S. mauritianum* berries. At this stage, the berries were approximately 1 cm in diameter.

For the callus initiation experiments, the berries used were obtained from various localities in Pietermaritzburg, but for the subsequent callus manipulation trials, all berries were obtained from the Muswell Hill region of Pietermaritzburg.

Berry explants were either placed singly on media in 25 by 100 mm culture tubes closed with Cap-O-Test lids and sealed with parafilm, or else, for the solasodine manipulation experiments, four explants were placed per screwtop glass jar. The tubes were filled with 10 or 15 ml of medium and the jars with 50 ml. The basic nutrient medium used was that of MURASHIGE and SKOOG (1962), modified to exclude glycine (Table 10), with 30 g l^{-1} sucrose and 0.1 g l^{-1} *myo*-inositol. The nutrients and vitamins were grouped and made up as stock solutions as indicated in Table 10. The media were adjusted to pH 5.8 prior to autoclaving. All media were solidified with 10 g l^{-1} agar (Unilab). Unless specified otherwise, all cultures were placed in a growth room with a low light intensity of 0.1 to 0.5 μmol m^{-2} s^{-1} and a temperature of 25 $\pm$ 1°C.

Table 10. Modified MURASHIGE and SKOOG (1962) medium used to initiate callus cultures from green berries of *S. mauritianum*.

Solution	Chemical	Mass (mg) per litre of medium
I	NH_4NO_3	1650
II	KNO_3	950
III	$CaCl_2$	344
IV	$MgSO_4.7H_2O$	370
V	NaFeEDTA	40
VI	KH_2PO_4	170
VII	H_3BO_3	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	KI	0.84
VIII	$NaMoO_4.2H_2O$	0.26
	$CuSO_4.5H_2O$	0.026
	$CoCl_2.6H_2O$	0.026
IX	Thiamine-HCl	0.10
	Nicotinic acid	0.50
	Pyridoxine-HCl	0.50

At the time of harvest, the pieces of callus in the bottles (four per bottle) were weighed and growth indices (final fresh weight - initial fresh weight / initial fresh weight) calculated. The callus was then pooled and a single extraction conducted per treatment. In order to determine solasodine levels in the callus, the samples were extracted and purified as described in Chapter 3, then benzoylated and injected

into the HPLC as given in Chapter 2. Solasodine standards were included with each set of samples benzoylated and thus standard curves set up, from which solasodine levels in the samples could be determined. Each sample was injected at least twice and the average peak area used to determine solasodine content. The samples were also spiked with solasodine standard to verify the identity of the solasodine peak. All results were ultimately expressed on a dry weight basis. Indications of callus productivity could be calculated from these values, since the initial number of bottles harvested and the total dry weight produced were known. The average moisture content of the callus following freeze-drying was calculated to be 96.16 $\pm$ 0.32 %.

5.3. Callus initiation: Results and Discussion

5.3.1. Sterilization test:

Various sterilization treatments were tested on the green *S. mauritianum* berries, following spraying of the berries with ethanol to remove any dirt and debris. The two sterilizing agents used were mercuric chloride at concentrations of 0.02 or 0.2 %, and commercial bleach (Jik). The latter contains approximately 3.5 % sodium hypochlorite and was used either at this strength or diluted to 1 % sodium hypochlorite. All solutions contained 2 drops of the surfactant, Tween 20. The solutions were applied for various time intervals, after which the berries were washed three times in sterile distilled water for 10 minutes at a time, with each wash consisting of three short rinses. The explants were examined for signs of contamination after one and three weeks (Table 11). Ten replicates were used per

treatment. The medium used was that of MURASHIGE and SKOOG (1962) modified as described and supplemented with 1 mg l^{-1} each of BA and NAA.

Table 11. Degree of sterility obtained following sterilization of green *S. mauritianum* berries under various conditions.

Treatment	Number of explants contaminated (out of ten)	
	Week 1	Week 3
0.02 % $HgCl_2$ 15 min	4	5
0.2 % $HgCl_2$ 10 min	0	0
1 % NaOCl 10 min	6	7
1 % NaOCl 20 min	3	5
3.5 % NaOCl 5 min	4	4
3.5 % NaOCl 10 min	0	0
3.5 % NaOCl 20 min	0	0

From Table 11, it can be seen that obtaining sterile explants from *S. mauritianum* berries did not pose a problem, with all explants in three treatments showing no signs of contamination after three weeks. The sterilization procedure could be relatively harsh, since the intact berries were sterilized and only the inner parts of the berries subsequently used as explants. It was elected to use 3.5 % sodium hypochlorite as sterilant rather than mercuric chloride, due to its lower human toxicity. A sterilization time of 15 minutes was chosen. This was the combination used in all subsequent trials requiring berry sterilization. This procedure was considerably simpler than that of JAGGI, BHATNAGAR, QADRY and KAPOOR (1987) to sterilize berries of *S. platanifolium* Sims. These authors first washed the

berries in running tap water, scrubbed them with detergent, washed them again in tap water, then distilled water, then sterilized them in 0.1 % mercuric chloride, rinsed them and further sterilized the berries by dipping them in alcohol and passing them over a flame. In *S. mauritianum*, all these precautions were not necessary.

During the course of the sterilization trial, it was noted that some callus formation had occurred on the surface of the explants after three weeks, indicating that MURASHIGE and SKOOG'S (1962) medium could be a suitable medium on which to initiate callus, as is the case for other *Solanum* species (Table 8). This was more apparent in the sodium hypochlorite trials than the mercuric chloride ones. The latter sterilizing agent may therefore be having a residual toxic effect on the explants.

5.3.2. Hormonal grid experiments:

Since a range of different hormones have been used for the initiation of callus from *Solanum* species (Table 8), a number of factorial grid experiments were conducted, in order to determine the most successful hormone combination for callus initiation from *S. mauritianum* explants. The auxins tested were naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indolebutyric acid (IBA), and the cytokinins, benzyladenine (BA) and kinetin. All were used at concentrations of 0, 0.5, 1 and 2 mg l^{-1} and all possible combinations were tested. These grid trials were conducted over a period of a year, through a range of different seasons (berries are available throughout the year; CAMPBELL and VAN STADEN, 1983),

with berries obtained from different areas in Pietermaritzburg. Therefore, the different trials could not be compared absolutely and the results in the controls (no hormones added) varied slightly. It was felt, however, that general trends should become obvious. The berry explants were weighed 4 weeks after culturing and the growth index (final fresh mass - initial fresh mass / initial fresh mass; UDDIN and CHATURVEDI, 1979) calculated for each treatment. An increase in the growth index (GI) signifies increased growth. If all cultures are harvested after the same time period, which they were in the present study, the GI gives an indication of the growth rate. Within each hormone combination trial, the results were subjected to an analysis of variance and a multiple range test conducted. Fifteen replicates were used per treatment. The results are given in Figure 26.

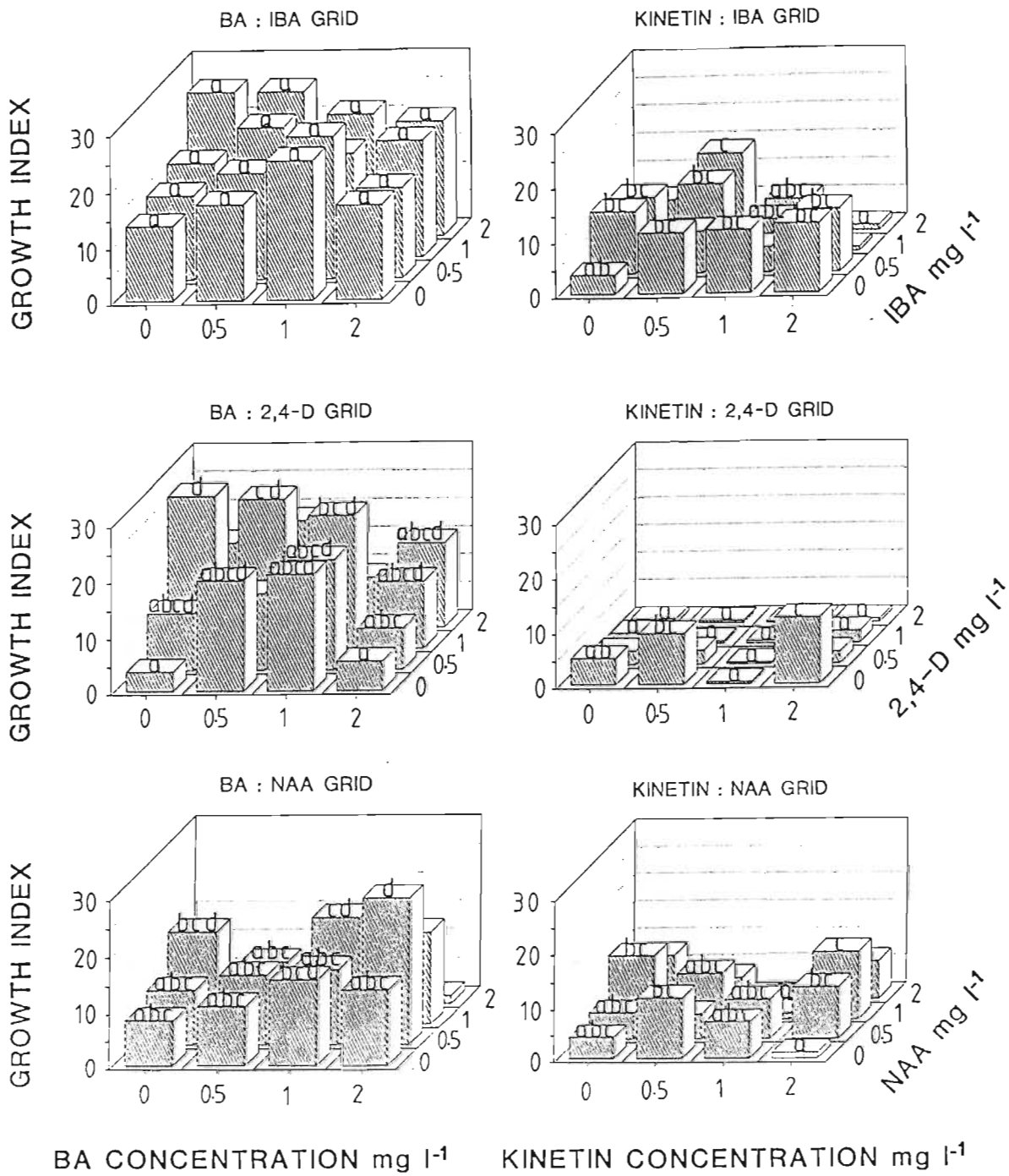

Figure 26. Growth indices (final fresh weight - initial fresh weight / initial fresh weight) of callus cultures of *S. mauritianum* four weeks after they were initiated from green berries using a variety of different hormone combinations. Within a treatment, columns with the same letter do not differ significantly at P = 0.05.

One fact that became apparent immediately was that callus could readily be initiated from berry explants, even in the absence of hormone supplements. In contrast, NIGRA, CASO and GIULIETTI (1987) had difficulty in initiating callus from *S. eleagnifolium* Cav. berries. Although callus growth of the *S. mauritianum* controls varied between trials, some was initiated in all instances. However, many hormonal treatments were found to increase callus growth beyond that of the controls. A notable exception was the combination of kinetin and 2,4-D, where growth was greatly reduced. Interestingly, in another report, in which *Solanum platanifolium* Sims berries were used as explants, it is precisely this combination of hormones that was found to promote callus growth greatly (JAGGI, BHATNAGAR, QADRY and KAPOOR, 1987). This combination has also been used for callus initiation from non-berry explants of *S. dulcamara* L. (EMKE and EILERT, 1986), *S. mammosum* L. (INDRAYANTO and SUTARJADI, 1986) and *S. laciniatum* Ait. (CHANDLER and DODDS, 1983). The hormone concentrations used in the reports were all within the range tested in the present study. Why the use of kinetin alone (Fig. 26) resulted in such low growth in this trial is unknown, since in the other trials it did yield adequate growth. Overall, however, it appears that the growth was less when kinetin was used in combination with the various auxins, than when BA was used. Subsequently, therefore, BA was the cytokinin of choice. Table 8 indicates that these two cytokinins have been used equally frequently in the initiation and maintenance of callus cultures of other wild *Solanum* species.

The auxin of choice for these species has been 2,4-D, but whether this is attributable to increased growth following its use, or whether it has just been used as a matter of course, has usually not been tested. In callus initiation from *S.*

mauritianum berries (Fig. 26), none of the three auxins tested appeared to be preferable to any of the others. In contrast, in *S. aculeatissimum* Jacq. stem explants, in which the efficacy of four auxins in the promotion of callus initiation was tested, 2,4-D and NOA were successful, whereas NAA and IAA resulted in no callus at all (GLEDDIE, KELLER and SETTERFIELD, 1985).

Although the growth index was at its overall maximum when a combination of IBA and BA was used, the control also yielded good growth in this case and there were no significant differences among all the concentration combinations tested. One trend that was evident (Fig. 26), was that use of 2 mg l^{-1} NAA in combination with BA was supraoptimal, growth being very low with all concentrations of BA.

Throughout all the grid trials conducted with *S. mauritianum*, only a few isolated tubes ever contained a root or a shoot. Therefore, if it were ever desired to propagate this species *vegetatively in vitro*, it seems it would be rather difficult to do so via callus. Indeed, MANDAL and GADGIL (1979) indicate fruit callus cultures to generally be recalcitrant with respect to organ induction. In some other *Solanum* species, however, for example *S. laciniatum* Ait. (CONNER, 1982), shoot initiation occurs readily from callus initiated from explants other than berries.

For subsequent initiation and maintenance of *S. mauritianum* callus, a combination of 2 mg l^{-1} BA and 1 mg l^{-1} NAA was chosen. This combination yielded growth in excess of that of the controls. In selecting this combination, the negative correlation between growth and secondary product synthesis was borne in mind. It was for this reason that the hormone combinations yielding the highest growth

were not selected. Subsequently, a new line of callus was initiated and maintained on this medium in bottles. Every four to six weeks it was subcultured onto fresh medium. Figure 27 shows a bottle of such four-week-old callus. When held under the low light conditions described, the callus was a creamy-white colour. It was also very friable, although sometimes small hard lumps were present.

Figure 27. Callus initiated from green *S. mauritianum* berries and maintained on a modified MURASHIGE and SKOOG (1962) medium containing 2 mg l^{-1} BA and 1 mg l^{-1} NAA.

5.4. Callus manipulation: Results and Discussion

In all trials attempting to manipulate *S. mauritianum* callus to produce solasodine, a basic MURASHIGE and SKOOG (1962) medium excluding glycine was used. Since callus could be initiated and grown in the absence of any hormones (Chapter 5.3.2), they were excluded from the medium, in order to keep the variables as few as possible.

5.4.1. Effect of light:

In most cases, callus cultures seem to be held in the dark or under low light conditions for growth (KOGA, SUESHIGE, TAHARA, KATO and IDE, 1990; BAUMERT, GRÖGER, KUZOVKINA and REISCH, 1992; NAIR, SUDHAKARAN, RAO and RAMAKRISHNA, 1992), although sometimes they are kept under ordinary laboratory lighting (DELFEL, 1980) or a 16 hours light : 8 hours dark regime (AGARWAL and BANSAL, 1989). On occasion, no indication of lighting is given at all. Despite the fact that callus cultures are usually grown in the dark or under low light conditions, the effect of light on the production of secondary metabolites in such systems has often been examined. The results obtained have been very varied. For example, in *Cinchona pubescens* Vahl., alkaloid production in callus cultures was stimulated by light (MULDER-KRIEGER, VERPOORTE, VAN DER KREEK and BAERHEIM-SVENDSEN, 1984), whereas in cocoa callus cultures, levels of theobromine and theophylline were significantly higher in the dark (GURNEY, EVANS and ROBINSON, 1992). No difference was observed between final levels of catechins in light and dark grown *Fagopyrum*

esculentum Moench. cultures, but the kinetics of the synthesis of one of the catechins differed (MOUMOU, VASSEUR, TROTIN and DUBOIS, 1992). The spectrum of secondary products synthesized under different light conditions can also vary. In *Cheilodonium majus* L. callus cultures, the dark-grown cells yielded a greater alkaloid variety than the light-grown cultures, on all three of the media that were examined (COLOMBO and TOME, 1991). Serpentine is the major alkaloid accumulated in the light in *Catharanthus roseus* (L.) G. Don. cultures, while ajmalicine is the major metabolite in the dark (MORRIS, 1986b). Overall, therefore, it can be concluded that the prevailing light conditions can definitely affect the synthesis of secondary products *in vitro*, but the type of response varies greatly.

Three possible light regimes were available for the present study. They were a constant low illumination (0.1 - 0.5 μmol m^{-2} s^{-1}), a continuous illumination with a higher light intensity (37 μmol m^{-2} s^{-1}) and a 16 hours light (62 μmol m^{-2} s^{-1}) : 8 hours dark regime. In all cases, the temperature was maintained at 25 $\pm$ 1°C. All the cultures were grown for 28 days prior to harvesting and determination of solasodine. The results are presented in Table 12. There was little difference in the amount of solasodine extracted from callus maintained under very low illumination and that in constant light, while levels were elevated in the 16 hours light : 8 hours dark treatment in which the light intensity was greatest.

Table 12. Solasodine content and growth of *S. mauritianum* callus grown under different light conditions.

Light conditions	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
Constant dim light ($0.1\ \mu mol\ m^{-2}\ s^{-1}$)	9.3	53.2 ± 5.6
Constant light ($37\ \mu mol\ m^{-2}\ s^{-1}$)	10.2	46.6 ± 3.0
16 h photoperiod ($62\ \mu mol\ m^{-2}\ s^{-1}$)	26.1	36.0 ± 2.8

During the course of this trial, it was noted that the callus in both the higher light intensity treatments was green in colour and in both, plantlets were present in some bottles (Fig. 28).

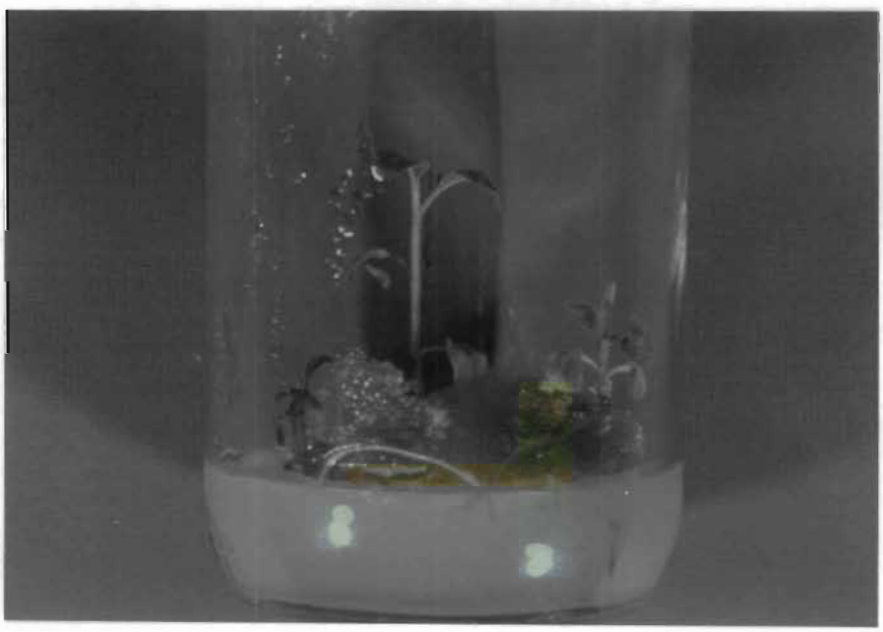

Figure 28. Plantlet initiation from *S. mauritianum* callus grown under constant illumination ($37\ \mu mol\ m^{-2}\ s^{-1}$) or a 16 hour photoperiod ($62\ \mu mol\ m^{-2}\ s^{-1}$).

Because of the frequently observed correlation between secondary product synthesis and differentiation (Chapter 4.3), it had been expected that both these two light treatments might result in solasodine levels elevated beyond those in the low light regime, in which the callus was creamy-white with no differentiated organs present. This was not the case (Table 12). Therefore, the degree of differentiation did not greatly affect solasodine accumulation. Perhaps it is rather a certain threshold light intensity (between 37 and 62 μmol m^{-2} s^{-1}) that must be attained in order for solasodine levels to be affected.

In a very interesting study, BHATT, BHATT and SUSSEX (1983) examined the solasodine content of callus of *S. nigrum* L. and the differentiated structures initiated from the callus, separately, under conditions of dark and a 16 hour photoperiod. They found that in the callus itself, more solasodine was present in the light, whereas in the roots and shoots derived from the callus, greater levels were found in the dark-grown cultures. Extraction of callus plus shoots may therefore give misleading results. In the present study, however, the level of morphogenesis was far too low to allow for extraction of the organs individually and would therefore also be unlikely to affect solasodine content significantly.

The greening of the tissue, which was observed in all bottles other than those held in dim light, has often been associated with a low growth rate of the tissue (COLLIN, 1987). Indeed, in *S. mauritianum* cultures, the cultures grown under very low light conditions and showing no greening did have the highest growth index (Table 12). In fact, negative correlation between growth rate and secondary product synthesis does seem to apply in this case, since the highest levels of

solasodine were extracted from the callus held under the highest light intensity and this callus exhibited the least growth. The productivity of this callus (micrograms of solasodine per bottle), was approximately double that of the low light intensity treatment, the increase in solasodine content more than compensating for the reduction in callus growth. This level of solasodine approached that previously found in the roots and leaves of a young *S. mauritianum* plant (Table 7, Chapter 3.3.5), but was far lower than the 184 $\mu g\ g^{-1}$DW recorded in the green berries.

The effect of light on solasodine production has been examined in callus cultures of several other *Solanum* species. In *S. laciniatum* Ait. (CONNER, 1987) and *S. eleagnifolium* Cav. (NIGRA, ALVAREZ and GIULIETTI, 1989), solasodine levels were also found to be greater in the light than in the dark. However, CHANDLER and DODDS (1983), who examined *S. laciniatum*, extracted more solasodine from cultures grown in the dark than from those in the light. In both these reports dealing with *S. laciniatum*, the callus was leaf-derived, but the hormone supplement in the media differed. Also, in the report of CONNER (1987), the light was continuous, whereas in that of CHANDLER and DODDS (1983), a 16 hour photoperiod was used. Light sources, quality and quantity are generally regarded as very variable factors in such studies (KURZ, 1986). This highlights the problem of making comparisons between different studies, in order to establish general trends of response, even if the same species is used. Each result appears to be specific to a certain species under a particular set of conditions, with slight alterations in the conditions resulting in significant differences. That light does affect solasodine accumulation in callus cultures does, however, seem to be indisputable.

5.4.2. Effect of gelling agent:

This is a factor that has not generally been examined with respect to its effect on secondary product synthesis *in vitro*, since agar has been used universally in the past. However, a water-soluble polysaccharide produced by a *Pseudomonas* species has now been developed as an alternative (KANG, VEEDER, MIRRASOUL, KANEKO and COTTRELL, 1982) and has been used successfully in bacteriological media (SHUNGU, VALIANT, TUTLANE, WEINBERG, WEISSBERGER, KOUPAL, GADEBUSCH and STAPLEY, 1983). It is commonly known as Gelrite. It has been reported (ICHI, KODA, ASAI, HATANAKA and SEKIYA, 1986) that callus growth and shoot propagation of a number of plants is greater on a Gelrite-containing medium than on an agar-containing one. Also, MACRAE and VAN STADEN (1990) found that in *Eucalyptus grandis* L., shoot multiplication and elongation, as well as rooting, were superior on a Gelrite-containing medium, than when agar was used as the gelling agent. Since growth and secondary product synthesis appear to be associated in some way, it is conceivable that the use of Gelrite rather than agar could have an effect on secondary product synthesis in *S. mauritianum* callus.

Berries were cultured on the medium described previously, but the media were solidified either with 10 g l^{-1} agar or 2 g l^{-1} Gelrite (Labretoria). The cultures were held under constant dim illumination and harvested after 28 days. The results are presented in Table 13.

Table 13. Solasodine content and growth of *S. mauritianum* callus initiated and grown on media gelled with agar or Gelrite.

Gelling agent	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
Agar	12.0	55.2 $\pm$ 3.5
Gelrite	10.4	38.7 $\pm$ 2.6

Very little difference was found in solasodine content, despite the fact that, surprisingly, callus growth was actually reduced when Gelrite was used. In this case, therefore, growth and solasodine synthesis were not negatively correlated, as was the case with the light treatments. Solasodine yield could not be enhanced by these means.

5.4.3. Effect of nutrients:

A wide range of manipulations of the nutrient medium have been conducted in the past in order to determine their effect on the production of secondary metabolites *in vitro*. CONSTABEL (1988) indicated that no other culture condition affects the performance of cells more than the composition of the medium.

In MURASHIGE and SKOOG's (1962) medium, the nutrients can be divided into three groups, namely the macro- and micronutrients, and the organic component, the vitamins. Commonly, the phosphate and nitrogen complements of the macronutrients are manipulated. Limitation of phosphate has been used to increase product yield in cultures (COLLINGE, 1986). It is thought that important enzymes linking primary to secondary metabolism are only activated when phosphate levels

are sufficiently low (KURZ, 1986). Therefore, a trial was conducted in which the level of phosphate (as KH_2PO_4) was reduced by a factor of five in the medium. At this level, growth of the culture should be limited (OKAZAKI, HINO, NAGASAWA and MIURA, 1982). The outcome in *S. mauritianum* callus cultures is given in Table 14.

Table 14. Solasodine content and growth of *S. mauritianum* callus cultures initiated and grown under normal and phosphate-limited conditions.

Phosphate level (KH_2PO_4 in mg l^{-1})	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
170	10.4	46.2 $\pm$ 3.8
34	25.2	6.9 $\pm$ 0.6

Although the solasodine extracted was greater (on a per gram basis) when phosphate was limited, the growth was severely retarded. Similarly, in *S. laciniatum* Ait. callus cultures, phosphate limitation severely affected growth of the culture, while accompanying increased solasodine concentrations were recorded (CHANDLER and DODDS, 1983). This is not a practicable means of increasing solasodine content of *S. mauritianum* cultures, since if the total amount of callus produced in the two trials over the same period is compared, the overall quantity of solasodine extractable is far greater in the non-phosphate-limited callus trial. Perhaps a less drastic decrease in phosphate levels would have been more effective, by maintaining a reasonable degree of growth and still increasing solasodine content. In a suspension culture of *Nicotiana tabacum* L., which is also a solanaceous species, the same degree of phosphate limitation did not result in such a marked reduction in growth and scopoletin levels increased (OKAZAKI, HINO, NAGASAWA and

MIURA, 1982). In contrast, in *Anchusa officinalis* L. (DE-EKNAMKUL and ELLIS, 1985) and *Galium vernum* Scop. (STROBEL, HIEKE, GEBAUER, WIND and GRÖGER, 1990), it was elevated levels of phosphate that were found to increase levels of the relevant secondary products. This is contrary to the general trend. This was particularly unexpected in *Anchusa*, since depletion of phosphate from the medium had been found to coincide with the onset of rosmarinic acid formation (DE-EKNAMKUL and ELLIS, 1985). Therefore, how phosphate actually affects secondary product synthesis remains unclear, although it definitely seems to have an effect.

Both the type and concentration of nitrogen in nutrient media have frequently been altered to observe their effects on secondary metabolite synthesis *in vitro* (see Chapter 1.4). In particular, the carbon : nitrogen (C : N) ratio appears to be important (KURZ, 1986). It was therefore elected to determine the effect of halving and doubling the usual C : N ratio, on the accumulation of solasodine in *S. mauritianum* callus cultures. To do this, the carbon source (sucrose) was kept at the same level (30 g l^{-1}), while the amounts of ammonium- and potassium nitrate were halved (to double the C : N ratio) or doubled (to halve the C : N ratio). All other variables were as described and after 28 days, the cultures were harvested and solasodine content determined (Table 15). The levels of solasodine extracted (Table 15) did not vary much in any of the treatments, although callus growth was considerably reduced when the ratio of C : N was doubled (low nitrogen).

Table 15. Solasodine content and growth of callus cultures of *S. mauritianum* initiated and grown on media containing different C : N ratios.

C : N ratio	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
0.5 x C : N	9.6	42.4 $\pm$ 3.7
1 x C : N	12.0	55.2 $\pm$ 3.5
2 x C : N	12.9	27.3 $\pm$ 1.6

It has been suggested (SUZUKI, MATSUMOTO and MIKAMI, 1984) that a reduction in nitrogenous nutrients may cause a switch of cellular metabolism to reactions forming nitrogen-free products. Since solasodine is a nitrogen-containing compound, this could imply a decrease in solasodine with a decrease in inorganic nitrogen (increased C : N ratio). This was, however, not found to be the case in *S. mauritianum* where solasodine levels remained unaffected by changes in nitrogen. In tobacco, the production of scopolin did seem to be regulated by the ratio of carbon to nitrogen. At 3 % sucrose, the amount of scopolin in the cells increased greatly with an increase in inorganic nitrogen (OKAZAKI, HINO, NAGASAWA and MIURA, 1982). Similarly, in *Cephalotaxus harringtonia* (Forbes) K. Koch callus cultures the presence or absence of ammonium nitrate affected alkaloid synthesis, suppressing certain compounds and promoting the production of others (DELFEL, 1980).

The next component of the medium whose effect on solasodine production in *S. mauritianum* callus was examined, was the micronutrient complement. This concerns the levels of manganese, zinc, copper, cobalt, iodine and boron. The

effect of adding these at half or quarter of their strength in a standard MURASHIGE and SKOOG (1962) medium was tested. The results are shown in Table 16.

Table 16. Solasodine content and growth of *S. mauritianum* callus initiated and grown on media containing a reduced micronutrient complement.

Level of micronutrients	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
0.25 x M & S	11.2	55.8 $\pm$ 5.1
0.50 x M & S	11.4	54.1 $\pm$ 2.9
1 x M & S	12.0	55.2 $\pm$ 3.5

The level of micronutrients was found to affect neither the solasodine content of the callus, nor its growth. In contrast, normal MURASHIGE and SKOOG (1962) levels of micronutrients have been found to actually reduce the amounts of most of the alkaloids present in *Cephalotaxus harringtonia* (Forbes) K. Koch callus cultures (DELFEL, 1980).

An extension of this trial was conducted by determining the effect of reducing the concentration of both the micro- and the macronutrients and the vitamins. The levels were again reduced to half and quarter their strength in a standard MURASHIGE and SKOOG (1962) medium and the callus harvested after 28 days (Table 17).

Although growth was reduced by the decrease in macro- and micronutrients, the levels of solasodine were increased greatly in both cases (Table 17), particularly

when a half-strength salt concentration was used. The solasodine productivity of this medium was 13.9 μg per bottle over four weeks, as opposed to a value of 4.6 when a full-strength MURASHIGE and SKOOG (1962) medium was used. The amount of solasodine produced therefore more than compensated for the reduction in callus growth. The use of a half-strength medium is therefore a suitable means of manipulating *S. mauritianum* callus to become more productive with respect to solasodine synthesis.

Table 17. Solasodine content and growth of *S. mauritianum* callus initiated and grown on media containing reduced levels of MURASHIGE and SKOOG (1962) salts and vitamins.

Level of nutrients	Solasodine content (μg g^{-1}DW)	Growth index ($\pm$ S.E.)
0.25 x M & S	47.2	15.5 $\pm$ 0.9
0.50 x M & S	64.5	20.3 $\pm$ 1.8
1 x M & S	9.3	53.8 $\pm$ 5.0

Medium strenght is a factor that has not generally been tested with respect to secondary product synthesis *in vitro*, and which the present results indicate is worth investigating. The stress induced by the reduction in the medium strength definitely appears to affect solasodine accumulation in *S. mauritianum* callus in some way. In *Duboisia leichhardtii* F. Muell. root cultures, however, no remarkable difference was found in alkaloid production when the comparative strength of the basal medium was altered (KITAMURA, SUGIMOTO, SAMEJIMA, HAYASHIDA and MIURA, 1991). It was thought that the effect in *S. mauritianum* callus cultures might be attributable to the reduction in the vitamin concentration,

since in root cultures of *Linum flavum* L. removal of vitamins resulted in an increase of 5-methoxypodophyllotoxin (VAN UDEN, PRAS, HOMAN and MALINGRÉ, 1991). This effect is not universal, however, since in suspension cultures of *Hyoscyamus muticus* L., a solanaceous species, no alkaloids were detected in cultures growing on vitamin-free media (KOUL, AHUJA and GREWAL, 1983).

5.4.4. Effect of carbohydrates:

The carbohydrate complement is a very important organic component of any tissue culture medium, being essential in heterotrophic cultures. The carbohydrate source may affect both differentiation and secondary metabolism, as well as callus growth and morphology (ALLAN, 1991). Glucose and sucrose usually result in the highest growth rate, but sucrose has generally been found to be preferable for secondary metabolite production (KURZ, 1986). The concentration of sucrose can greatly influence cell growth and secondary metabolism and therefore, a trial was initiated in which the medium was supplemented with 2, 3, 4 or 5 % sucrose, the usual level being 3 %. However, after 28 days, the little callus that had formed in the 4 and 5 % sucrose trials had senesced and was therefore not extracted. This trial was repeated and the outcome was unchanged. Elevated sucrose levels therefore seem to be detrimental to *S. mauritianum* callus growth. Similarly, in *Fagopyrum esculentum* Moench. callus cultures, if sucrose concentrations were greater than 4 %, growth decreased and browning occurred (MOUMOU, TROTIN, DUBOIS, VASSEUR and EL-BOUSTANI, 1992). This response was also apparent in suspension cultures of *Achillea millefolium* L., in which growth decreased and

oxidation was observed after several subcultures when elevated sucrose concentrations were used (FIGUEIREDO and PAIS, 1991). In another *Solanum* species, *S. laciniatum* Ait., an increase in sucrose concentration beyond 3 % resulted in a decrease in the fresh weight of callus cultures, without browning, and an accompanying increase in solasodine production (CHANDLER and DODDS, 1983). The effect on callus growth was not nearly as marked as in *S. mauritianum*.

This detrimental effect of increased sucrose concentrations on callus growth is by no means universal. In *Duboisia* (KITAMURA, SUGIMOTO, SAMEJIMA, HAYASHIDA and MIURA, 1991) and *Linum flavum* L. (VAN UDEN, PRAS, HOMAN and MALINGRÉ, 1991) root cultures and cell suspension cultures of *Galium vernum* Scop. (STROBEL, HIEKE, GEBAUER, WIND and GRÖGER, 1990), elevated levels of sucrose resulted in increased biomass production and an increase of the relevant secondary metabolites. The reverse relationship was observed in *Hyoscyamus muticus* L. in which, although growth was maximal at increased sucrose levels, scopolamine production was at a low level and only increased when the amount of sucrose was decreased towards 0 % and growth was minimal (OKSMAN-CALDENTEY, STRAUSS and HILTUNEN, 1986). This contradicts the theory of KURZ (1986) who postulated that if the carbohydrate levels are too low, the available substrate will be used entirely for cell growth and not for secondary metabolite production. Therefore, depending on the species involved and the product concerned, results appear to differ widely.

Although sucrose is generally the most suitable carbohydrate for cell cultures, many cultures can assimilate other carbon sources (DE-EKNAMKUL and ELLIS, 1985). The effect of using glucose and fructose, the hydrolysis products of sucrose, directly, as sources of carbohydrate, on the synthesis of solasodine in callus cultures of *S. mauritianum* was tested. For this purpose, treatments were set up containing 3 % sucrose, glucose or fructose, 5 % glucose or fructose, or a combination of 1.5 % each of glucose and fructose. This latter treatment is equivalent to 3 % sucrose, if complete hydrolysis were to occur, whereas in the others, the levels of the specific reducing sugars are greater than usual. The results are presented in Table 18.

Table 18. Solasodine content and growth of callus of *S. mauritianum* initiated and grown on media containing different amounts and types of carbohydrates.

Carbohydrate source	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
1.5 % glucose + 1.5 % fructose	7.4	53.4 $\pm$ 4.7
3 % sucrose	8.5	49.9 $\pm$ 4.1
3 % glucose	31.4	67.3 $\pm$ 5.7
3 % fructose	-	61.9 $\pm$ 5.2
5 % glucose	55.8	35.6 $\pm$ 3.1
5 % fructose	-	40.3 $\pm$ 3.0

When the combination of glucose and fructose was used, both growth and solasodine production were very similar to when 3 % sucrose was included in the medium. Therefore, the hydrolysis of sucrose into glucose and fructose, in which form the sugars are thought to enter the cells, appears to be efficient. Similarly,

in carrot (ZWAYYED, FRAZIER and DOUGALL, 1991) and *Anchusa officinalis* L. (DE-EKNAMKUL and ELLIS, 1985) cell suspensions these two treatments were indistinguishable as regards growth and secondary product synthesis.

In contrast, when glucose or fructose alone were used at 3 % (Table 18), callus growth was increased beyond that of the 3 % sucrose control. In the absence of glucose, however (fructose only), no trace of solasodine was detected at all, whereas with 3 % glucose, solasodine levels were elevated beyond those of the control. This trend was even more marked when these sugars were used at 5 %, although at this level, growth of the callus was reduced slightly. The presence of glucose therefore appeared to stimulate solasodine synthesis, whereas in the presence of fructose only, no solasodine synthesis was apparent, although callus growth was unaffected. This implied that when sucrose was used as the carbohydrate source, following hydrolysis, it was only the glucose that affected solasodine synthesis, the fructose being superfluous for this purpose.

The solasodine productivity of the callus was 24.3 μg per bottle over 28 days with 5 % glucose and 15.5 μg per bottle with 3 % glucose, compared to only 3.5 μg per bottle when 3 % sucrose was used. Both of the glucose treatments therefore considerably increased the solasodine productivity of the system. Whether the involvement of glucose is direct, or whether it impinges on some metabolic process and so influences solasodine synthesis, is unknown. A means of determining this would be to feed callus with radiolabelled glucose and follow its incorporation. This presented a problem in the present study, however, since only free solasodine and not its glucosylated forms are detectable with the HPLC technique developed.

It is possible, that the effect of glucose is direct, since glucose is part of the triose sugar moiety of both solasonine and solamargine. These are the glucosylated forms of solasodine in which solasodine is most commonly found in plants (OSMAN, 1980). However, it is likely that the other component sugars of the triose moieties, namely galactose and rhamnose, would also have to be supplied at elevated levels for the stimulation of solasodine synthesis by glucose to be direct. The effect is therefore more likely to be indirect.

In contrast to the trend in *S. mauritianum* callus cultures, in batch suspension cultures of *S. eleagnifolium* Cav., it was found that an increase in glucose concentration did not affect solasodine yield, whereas it was increased with an elevated sucrose concentration (NIGRA, ALVAREZ and GIULIETTI, 1990). Therefore, even though the secondary product of interest may be the same as in the present study, the response is different in a different culture type of another *Solanum* species. The effect of different sugars at various concentrations has been examined on the synthesis of other glycosidic secondary products, such as anthocyanins. For example, increased glucose resulted in better anthocyanin accumulation than fructose in carrot suspension cultures (ZWAYYED, FRAZIER and DOUGALL, 1991), which mirrors the situation in *S. mauritianum* callus.

The effect of monosaccharides on the synthesis of various non-glycosidic secondary metabolites has also been tested. Fructose has been found to enhance catharanthine yield in *Catharanthus roseus* (L.) G. Don. hairy root cultures, while reducing growth, relative to sucrose. It was suggested that the use of fructose alters carbohydrate metabolism in hairy roots in some way, and this subsequently

stimulates their secondary metabolism (JUNG, KWAK, KIM, LEE, CHOI and LIU, 1992). In contrast, while fructose and glucose decreased growth of *Panax ginseng* C. A. Meyer root cultures, maximum ginsenoside content was recorded with fructose or sucrose (ODNEVALL and BJÖRK, 1989). Fructose was found to be as effective as sucrose for both growth and rosmarinic acid formation in *Anchusa officinalis* L., while glucose was slightly inferior on both accounts (DE-EKNAMKUL and ELLIS, 1985). Neither glucose nor fructose affected the growth of cells of a tobacco suspension, but the formation of scopoletin decreased in both treatments, relative to the sucrose treatment (OKAZAKI, HINO, NAGASAWA and MIURA, 1982). Overall, therefore, the effects of the different carbohydrates are very varied with respect to growth and secondary product synthesis in culture, and, even when uptake kinetics and carbohydrate utilization within the cells have been examined, no conclusive reasons have been found to account for the different responses.

5.4.5. Effect of hormones:

The quality and quantity of growth regulators in media are very important factors, since the synthesis and accumulation of secondary products in plant cells appears to be a function of both growth and differentiation. Plant hormones do not react with intermediates, as other medium components do, but seem to be able to shift conditions in favour of product formation. Their action is usually complex and the formation of several metabolites can be affected by the same hormone (KURZ, 1986). Growth trials of *Solanum mauritianum* have already been conducted with a wide range of hormone combinations (Section 5.3). The BA : NAA grid was

selected to examine the effect of hormones on solasodine synthesis in these cultures. The range of concentrations chosen had previously yielded differing degrees of growth (Fig. 26). The use of 2 mg l^{-1} NAA, which had been found to reduce callus growth greatly, was avoided. The concentrations ultimately used and the levels of solasodine extracted from the callus that subsequently developed, are given in Table 19.

Table 19. Solasodine content and growth of *S. mauritianum* callus initiated and grown on a medium containing a variety of different combinations of BA and NAA.

Concentration of BA : NAA (mg l^{-1})	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index ($\pm$ S.E.)
0 : 0	10.2	55.1 $\pm$ 3.6
0 : 1	10.1	44.5 $\pm$ 4.2
1 : 0.5	10.6	60.3 $\pm$ 3.9
1 : 1	25.4	70.4 $\pm$ 5.6
2 : 0.5	33.4	58.2 $\pm$ 5.3
2 : 1	69.7	60.5 $\pm$ 5.9

The addition of no BA and 1 mg l^{-1} NAA did not affect solasodine accumulation at all, relative to the hormone-free control. There did, however, seem to be some form of interaction occurring when both BA and NAA were used, since the addition of 1 mg l^{-1} BA increased solasodine levels when 1 mg l^{-1} NAA was present, but not with only 0.5 mg l^{-1} NAA. This same trend was apparent when the level of BA was increased to 2 mg $^{-1}$, with either 0.5 or 1 mg l^{-1} NAA. Elevated levels of BA seemed to result in accumulation of considerably increased levels of solasodine, but

this was influenced by the amount of NAA present, although on its own, NAA had no effect. The hormone complement does therefore appear to be important in the regulation of solasodine synthesis *in vitro*. None of the hormone combinations tested were found to be detrimental to solasodine production, while callus growth was frequently greater than in the control, making these highly productive media.

The reports of hormonal effects on secondary product synthesis *in vitro* are very varied. Some other *Solanum* species have been examined. In *S. eleagnifolium* Cav. suspension cultures, simultaneous administration of auxin and cytokinin was also found to increase solasodine productivity, although growth was reduced (ALVAREZ, NIGRA and GIULIETTI, 1993). This was calculated relative to an auxin only control. A similar trend of increased solasodine synthesis was observed in *S. laciniatum* Ait. (HOSODA, ITO and YATAZAWA, 1979) and in callus of the solanaceous *Nicotiana tabacum* L., more scopolin and scopoletin were produced when the hormones BA and NAA were used together, than when either was used alone (HINO, OKAZAKI and MIURA, 1982; OKAZAKI, HINO, KOMINAMI and MIURA, 1982). Auxins were found to affect solasodine synthesis in *S. xanthocarpum* Schrad. et Wendl. cultures, where no solasodine was detectable when IAA or IBA were used as auxins, but it was present when 2,4-D was added (HEBLE, NARAYANASWAMY and CHADHA, 1971). In general, the effect of auxins has been examined more widely than that of cytokinins. Elimination of auxin from the medium of root cultures of two *Hyoscyamus* species, four *Datura* species, *Atropa belladonna* L. and *Duboisia leichhardtii* F. Muell., all solanaceous species, reduced the growth rate, but increased the tropane alkaloid of most of the cultures (HASHIMOTO, YUKIMUNE and YAMADA, 1986). In contrast, the

addition of a range of auxin and cytokinin combinations at various concentrations could not alter the pattern of secondary metabolite production in callus cultures of *S. platanifolium* Sims (JAGGI, BHATNAGAR, QADRY and KAPOOR, 1987).

This is unusual, in that in most instances, auxins and cytokinins do affect secondary product synthesis in some way and the way in which it is affected varies greatly between related species. This does not only apply to solanaceous species, but occurs in most plant families. For example, in the genus *Thalictrum* (Ranunculaceae), the production of the isoquinoline alkaloid, berberine, has been examined in the suspension cultures of three species. In *T. dipterocarpum* Franch. and *T. minus* L., berberine synthesis was markedly stimulated by a combination of BA and NAA, whereas the same auxin suppressed it in *T. flavum* L. (NAKAGAWA, FUKUI and TABATA, 1986; SUZUKI, NAKAGAWA, FUKUI and TABATA, 1988). Members of the Rubiaceae, which produce anthraquinones, have also been examined. In cells of *Rubia cordifolia* L., low concentrations of NAA increased anthraquinone content when compared to IAA and 2,4-D (SUZUKI, MATSUMOTO and MIKAMI, 1984). In another member of the Rubiaceae, *Cinchona succirubra* Pavon ex Klotzsch, it was increasing concentrations of either NAA or IAA that were found to increase cell growth and anthraquinone production, while increasing concentrations of 2,4-D inhibited both parameters (KHOURI, IBRAHIM and RIDEAU, 1986). Overall, therefore, no general rule can be applied in order to predict the possible effect of any hormones on secondary product synthesis, and the optimal type and concentrations must be determined individually for each species. This empirical approach may seem laborious, but until more is known of the exact mechanisms by which hormones

affect secondary product synthesis, there is no alternative.

5.4.6. Solasodine synthesis in an "optimal" medium:

A combination of the treatments individually found to be most favourable with respect to solasodine accumulation was tested, to determine whether solasodine levels could be elevated further. As a basis, full- and half-strength MURASHIGE and SKOOG (1962) media were both used, since, although the latter increased levels of solasodine in the callus considerably, growth of the callus was markedly reduced. To each of these media, 5 % glucose was added as carbohydrate source, together with 2 mg l^{-1} BA and 1 mg l^{-1} NAA as the hormone complement. The media were solidified with 1 % agar and subsequently the cultures were placed under a 16 hours light (62 μmol m^{-2} s^{-1}) : 8 hours dark light regime for four weeks. The results obtained after this time are given in Table 20.

Table 20. Solasodine content and growth of *S. mauritianum* callus initiated and grown on full- or half-strength modified MURASHIGE and SKOOG (1962) medium, with 5 % glucose, 2 mg l^{-1} BA and 1 mg l^{-1} NAA, under a 16 hour : 8 hour photoperiod.

Medium strength	Solasodine content (μg g^{-1}DW)	Growth index ($\pm$ S.E.)
1 x M & S	8.9	46.8 $\pm$ 5.1
0.5 x M & S	42.0	16.8 $\pm$ 2.1

When using full-strength MURASHIGE and SKOOG (1962) medium, solasodine content was no greater than that previously recorded with 3 % sucrose, no hormones and under low light conditions, the combination used as a control in the

earlier trials. Surprisingly, therefore, the stimulatory effects of the various treatments individually were by no means additive, but rather they were reduced relative to the treatments applied alone. With the use of half-strength MURASHIGE and SKOOG (1962) medium, however, the promotory effect of the individual treatment was maintained and solasodine levels were elevated in the callus, although never beyond those recorded for the treatments alone. Even when taking into account the severely reduced growth on half-strength MURASHIGE and SKOOG (1962) medium, this medium is still the most favourable for solasodine production, since the productivity on this medium is 11.45 μg solasodine per bottle, whereas on the full-strength medium, the value is 5.71 μg solasodine per bottle, over the same time period. Thus, overall, the use of a half-strength MURASHIGE and SKOOG (1962) medium, with sucrose or glucose, with or without hormones, can be regarded as a suitable medium for the production of elevated levels of solasodine in callus cultures of *S. mauritianum*, at this stage. Further manipulation of factors not tested in the present study, such as temperature and stress, and the careful selection of high-yielding lines, could potentially increase solasodine production further.

5.4.7. Maintenance of solasodine-producing ability through subculture:

In all the previously-described manipulations, callus was both initiated and grown on the different media and solasodine levels determined at the end of a four week incubation period. Some solasodine may in fact have been carried over from the original berry explants, which is why control cultures with a basic medium were initiated with each separate trial. The solasodine content of these control cultures

grown with the different trials actually varied very little, indicating that either the carry-over of solasodine was very consistent, or else the amount synthesized in these cultures was uniform. Since the use of fructose in the medium decreased solasodine to undetectable levels, however, this basic level recorded in the controls is likely to be a genuine value for solasodine synthesis.

If, however, the ability to synthesize solasodine *in vitro* is not sustainable through subculture, then the technique cannot be used as a means to produce solasodine on a continuous basis, and direct harvesting and extraction of the berries would be more feasible. In other species, secondary metabolite levels have frequently been found to decrease in callus cultures with time. For example, in *Duboisia leichhardtii* F. Muell. callus, tropane alkaloid content was found to decrease until the compounds were undetectable in successive subcultures (YAMADA and ENDO, 1984). Similarly, in *Digitalis purpurea* L. callus cultures, the presence of certain secondary metabolites decreased and was only evident up to the thirteenth subculture. Others, however, were still detectable after 29 subcultures (KARTNIG, KUMMER-FUSTINIONI and HEYDEL, 1983). In the same way, in cocoa leaf-derived callus, a reasonably constant production of purine alkaloids was maintained, although cotyledon-derived callus became less productive after one year (GURNEY, EVANS and ROBINSON, 1992).

In the present study, callus was initiated and maintained on a full-strength MURASHIGE and SKOOG (1962) medium containing 3 % sucrose with 2 mg l^{-1} BA and 1 mg l^{-1} NAA, following the initial hormone grid growth trials (Section 5.3.2). This was routinely subcultured every four to six weeks and at each

subculture, some callus was flash-frozen in liquid nitrogen, freeze-dried, ground and stored in airtight jars. The samples from the fifth, tenth, 15th and 20th subcultures were ultimately extracted to determine the solasodine levels following serial subculture. In addition, callus from the "optimal" medium trial (Section 5.4.6) was subcultured on both half- and full-strength MURASHIGE and SKOOG (1962) medium containing 5 % glucose, 2 mg l^{-1} BA and 1 mg l^{-1} NAA, and solasodine determined after the second, third and fourth subcultures. The results are presented in Table 21. For comparative purposes, the results obtained previously in the other trials where these media were used, are given as subculture one.

Table 21. Solasodine content of *S. mauritianum* callus following repeated subculture.

Medium	Subculture number	Solasodine content (μg g^{-1}DW)
1 x M & S	1	69.7
3% sucrose	5	13.5
2mgl^{-1} BA	10	-
1mgl^{-1} NAA	15	15.5
Low light	20	12.4
0.5 x M & S	1	42.0
16h : 8h photoperiod	2	28.2
5% glucose	3	67.3
2mgl^{-1} BA, 1mgl^{-1} NAA	4	21.7
1 x M & S	1	8.9
16h : 8h photoperiod	2	13.0
5% glucose	3	-
2mgl^{-1} BA, 1mgl^{-1} NAA	4	-

It can be seen that in the trial involving examination of 20 subcultures, solasodine levels initially dropped greatly, but then seemed to remain relatively constant. Even after 20 subcultures, solasodine was still detectable. Therefore, solasodine can be sustainably synthesized in *S. mauritianum* callus cultures. In the "optimal" media, synthesis did not appear to be sustained over four subcultures when full-strength MURASHIGE and SKOOG (1962) medium was used. In contrast, when the half-strength medium was used, although levels of solasodine fluctuated over the four subcultures examined, solasodine synthesis was maintained at a relatively high level throughout. Therefore, this system could well be used to synthesize solasodine on a continuous basis. In some other *Solanum* species, solasodine synthesis has also been found to be sustainable. For example, juvenile callus tissue of *S. laciniatum* Ait. has been observed to have the same solasodine-producing ability as callus tissues that have been cultured through more than 60 generations (HOSODA, ITO and YATAZAWA, 1979). From the point of view of sustainability therefore, such cultures are commercially viable, but it must be taken into account that callus growth is relatively slow and solasodine content low in these cultures.

5.5. Conclusions

It could be concluded that green berries of *S. mauritianum* were suitable explants for the initiation of callus cultures. The berries were easily sterilized and callus could be initiated and grown on a basic MURASHIGE and SKOOG (1962) medium containing 3 % sucrose, 0.1 g l^{-1} *myo*-inositol, with or without hormones. Certain hormone combinations did, however, stimulate callus growth beyond that obtained if exogenous hormones were lacking, for example 2 mg l^{-1} BA with 1 mg l^{-1} NAA.

Several factors seemed to increase the production of solasodine in *S. mauritianum* callus cultures, including light, the strength of the medium, the carbohydrate source and the hormonal complement. In some instances, the increase in solasodine content was accompanied by a decrease in callus growth, for example when phosphate was limited or when the salt and vitamin concentration was halved. However, this trend did not always apply, since when a basic medium was used with 2 mg l^{-1} BA and 1 mg l^{-1} NAA, both growth and solasodine content were elevated. Upon combining various factors that increased solasodine production when applied individually, no further increase in solasodine production was observed. Determination of solasodine levels during serial subcultures on certain media, indicated that solasodine synthesis could be sustained with time. When a half-strength medium was used, this level remained greater through subculture than that recorded at the first subculture of the controls. Overall, the highest levels of solasodine recorded in *S. mauritianum* callus tissues were approximately three times less than those recorded in the fresh green berries, but equivalent to those previously recorded in the vegetative parts of the plant (Chapter 3).

CHAPTER 6

SUSPENSION CULTURES OF *SOLANUM MAURITIANUM*

6.1. Introduction

Suspension cultures have frequently been established from economically important *Solanum* species. These have been developed for widely differing reasons, for example: examination of proline metabolism under conditions of water stress (CORCUERA, HINTZ and PAHLICH, 1989); production of diacetylenic phytoalexins (IMOTO and OHTA, 1988); examination of cytodifferentiation (REYNOLDS, 1987); and to provide a source of protoplasts for somatic cell fusion (KAMEYA, MIYAZAWA and TOKI, 1990). In contrast, relatively few reports exist of wild *Solanum* species from which suspension cultures have been initiated. In even fewer cases, has the solasodine content of the cultures been examined. A summary of some of these reports is given in Table 22.

Table 22. Wild *Solanum* species in which suspension cultures have been successfully initiated. Where examined, the solasodine content of these cultures is given.

Species	Medium	Hormones	Solasodine (mg g^{-1}DW)	Reference
S. xanthocarpum Clarke	Revised M&S (RT)	2,4-D	0.028	KHANNA, UDDIN, SHARMA, MANOT & RATHORE (1976)
S. jasminoides Paxt.	Revised M&S (RT)	2,4-D	1.1 - 2.0	JAIN & SAHOO (1981a)
6 wild species	M&S	2,4-D	not examined	GLEDDIE, KELLER & SETTERFIELD (1985).
S. dulcamara L.	M&S	2,4-D Kinetin	0.1 (total alkaloids)	EMKE & EILERT (1986)
S. carolinense L.	M&S	2,4-D Kinetin	not examined	REYNOLDS (1987)
S. aviculare Forst.	M&S	2,4-D Kinetin	not examined	VANĚK, MACEK, STRÁNSKY & UBIK (1989)
S. aviculare Forst.	M&S salts	NOA Kinetin	3.0	TSOULPHA & DORAN (1991)
S. eleagnifolium Cav.	Revised M&S (RT)	2,4-D or 2,4,5-T	0.91 - 1.0	ALVAREZ, NIGRA & GIULIETTI (1993)

Abbreviations: M & S: Medium of MURASHIGE and SKOOG (1962); RT: MURASHIGE and SKOOG (1962) salts with the RT vitamins of KHANNA and STABA (1968); 2,4-D: 2,4-dichlorophenoxyacetic acid; 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid; NOA: naphthoxyacetic acid.

As was observed with *Solanum* callus cultures, MURASHIGE and SKOOG (1962) medium appeared to be the basal medium of choice for *Solanum* suspension cultures. Again, the so-called RT vitamin supplement of KHANNA and STABA (1968) frequently replaced that of MURASHIGE and SKOOG (1962). Almost without exception, 2,4-D was the auxin of choice and in many instances it was the only hormone present in the medium. If cytokinins were also added to the medium, kinetin was the one most commonly chosen. The exception is the report of TSOULPHA and DORAN (1991) in which suspension cultures of *S. aviculare* Forst. were initiated and maintained on a MURASHIGE and SKOOG (1962) medium containing naphthoxyacetic acid as auxin and kinetin as cytokinin.

The solasodine content of *Solanum* suspension cultures, when determined, has usually been in the low milligram range (Table 22), which is slightly greater than the average previously observed in callus cultures (Table 9, Chapter 5). In most studies, solasodine content was only determined in the cells and not in the medium. When it was measured in the stale medium, levels were found to be low. For example, in *S. aviculare* Forst., 5 % of the total solasodine recorded was in the medium (TSOULPHA and DORAN, 1991) and with *S. xanthocarpum* Clarke, only a trace of solasodine was detected in the spent medium (KHANNA, UDDIN, SHARMA, MANOT and RATHORE, 1976). The cells do thus not release much solasodine. This suggests that if suspension cultures of *Solanum* species were to be bulked up for the extraction of solasodine, the systems would probably have to be batch cultures, not continuous systems.

In the examples of *Solanum* suspension cultures given in Table 22, the details of culture parameters, such as inoculum size, time between subcultures and rotation speed, varied greatly. In no instance was much difficulty reported in initiating the suspensions, which suggested that suspensions could probably be initiated from *S. mauritianum* callus fairly readily.

6.2. Materials and Methods

The callus used to initiate the suspensions of *S. mauritianum* had been regularly subcultured on a solid MURASHIGE and SKOOG (1962) medium (excluding glycine) for at least eight months, at the time of use. The callus medium was supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol, 2 mg l^{-1} BA and 1 mg l^{-1} NAA. The cultures were maintained under a continuous low light intensity (0.1 μmol m^{-2} s^{-1}) at a temperature of 25 $\pm$ 1°C. For the initiation of the suspension cultures, callus that had been subcultured four to six weeks previously, was used. If the inoculation density for suspension cultures is too low, they may not grow. Two to three grams of friable callus in 100 ml of medium has been recommended as a minimum density (SMITH, 1986), although frequently, amounts in the order of 20 g or more are used (NIGRA, ALVAREZ and GIULIETTI, 1990). Therefore, the exact weight of *S. mauritianum* callus used was not determined, but it was ensured that it was greater than 5 g. The volume of medium used was 100 ml throughout and this was contained in a 500 ml conical flask. The flasks were stoppered with cottonwool bungs, which were then covered with aluminium foil.

Unless stated otherwise, the suspension cultures were incubated in Gallenkamp orbital incubators with a controlled temperature of 25 $\pm$ 0.5°C and a continuous light intensity of 45 μmol m^{-2} s^{-1}. The platforms of the shakers were rotated at 150 rpm. For the initiation of the suspensions, the inoculated flasks were left to shake for two weeks, after which 50 ml fresh medium was added. After a further two weeks, a 25 % inoculum of the cultures was transferred into fresh medium in a sidearm flask and growth monitored. Upon transfer, most of the larger clumps of cells were left behind and thus excluded.

Growth of suspension cultures can be monitored by various means, for example, conductivity, settled cell volume, packed cell volume, fresh cell weight, optical density, protein, DNA and RNA measurements (RYU, LEE and ROMANI, 1990). All have various advantages and disadvantages. The technique chosen for the present study was that of settled cell volume (SCV). The results using this principle have been found to be highly correlated with the fresh volume of the cells in other species (BLOM, KREIS, VAN IREN and LIBBENGA, 1992).

In order to determine SCV, 500 ml conical flasks were used that had a sidearm attached (Fig. 29). The flask contents were swirled to distribute the cells evenly in the medium, the sidearms filled with the suspension and the flask clamped at such an angle that the upper portion of the sidearm was vertical. After 5 minutes, the flask was tilted until the lower part of the sidearm was vertical. After a further 5 minutes, the SCV was marked on the sidearm. The results were ultimately expressed as a percentage of the total volume of the sidearm. The advantage of this technique is that it is rapid, with careful timing several flasks can be monitored

simultaneously, it is non-destructive and the culture is not opened and thus exposed to potential sources of contamination.

Figure 29. Example of a 500 ml sidearm flask in which growth of the *S. mauritianum* suspension cultures was monitored.

In order to determine viability of the cultures, the fluorescein diacetate (FDA) technique of WIDHOLM (1972) was used. The principle of staining with this chemical relies on the non-polar FDA molecule crossing the plasma membrane and its ester bonds being hydrolyzed in the cytoplasm to release fluorescein. The polar fluorescein molecule cannot pass through the plasma membrane or tonoplast of living cells (HESLOP-HARRISON and HESLOP-HARRISON, 1970). Living cells are therefore distinguished by their bright fluorescence when illuminated with blue light (HUANG, CORNEJO, BUSH and JONES, 1986). A stock solution of 0.5 % fluorescein diacetate (FDA) in acetone was stored in a deep-freeze. When required, aliquots were removed and one drop added per 10 ml of diluted (1:10) suspension. Samples were then pipetted onto microscope slides and after 5 minutes were

examined under ultra-violet light using a Zeiss IM35 inverted microscope for transmitted light. By including some white light, both living and dead cells could be observed simultaneously (Fig. 30).

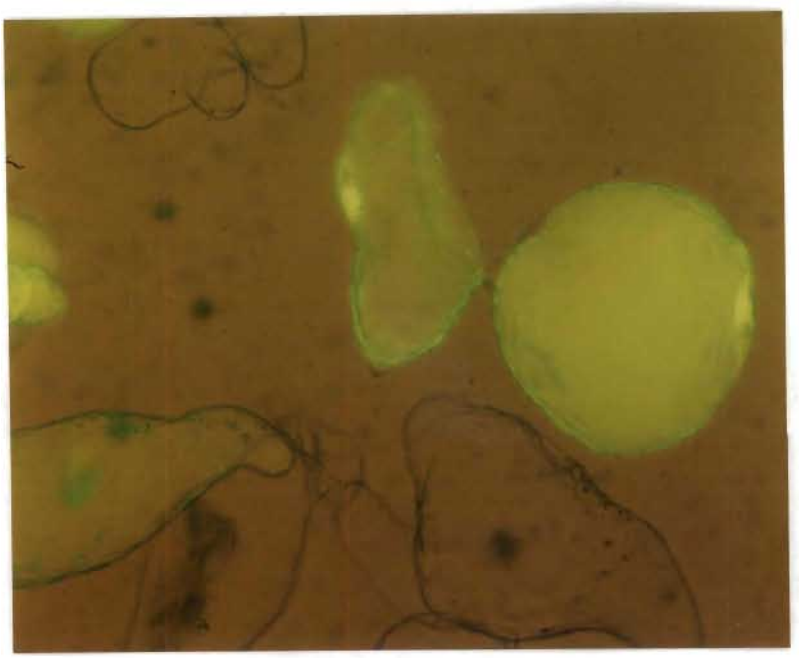

Figure 30. Living (fluorescing) and dead (non-fluorescing) cells of a *S. mauritianum* suspension culture stained with fluorescein diacetate and viewed under a mixture of visible and ultraviolet light.

For subculturing of the suspensions, large clumps of cells were allowed to settle out and the rest of the sample poured into a 100 ml measuring cylinder. After 10 minutes, the spent medium and any excess settled cell volume were decanted. Fresh medium was added to the remaining, measured volume of cells to make a total volume of 100 ml. This solution was then swirled and carefully poured into sterile flasks. The cultures were then returned to the shakers.

At the time of harvest, for the purpose of solasodine determination, the cultures were filtered through Whatman No. 1 filter paper using a Büchner funnel. The

cells were then weighed, flash-frozen in liquid nitrogen and freeze-dried. The spent medium was reduced to a minimum *in vacuo*, redissolved in 80 % ethanol and stored in a deep-freeze until required for analysis. The samples were then extracted, purified, benzoylated and solasodine levels determined by HPLC, as described in Chapters 2 and 3. The results were ultimately calculated on a per gram dry weight basis for purposes of comparison.

6.3. Initiation of suspensions: Results and Discussion

STREET (1977) described the choice of growth medium as one of the most confusing aspects of work with suspension cultures. To maximize product yield, this medium must contain an appropriate balance of nutritional and hormonal components to allow full expression of the metabolic pathways related to secondary product biosynthesis (SMITH, QUESNEL, SMART and MISAWA, 1987). The callus of *S. mauritianum* was very friable and it therefore seemed likely that it would break up easily in a shaken liquid medium to form a suspension.

6.3.1. Use of callus growth medium:

The logical medium in which first to attempt to initiate suspensions of *S. mauritianum* was a liquid form of that on which the callus had been growing very prolifically. ALLAN (1991) indicated that in most instances, callus and suspensions of one species can be grown on the same medium, although this is not always the case. The medium tested in the present trial was therefore that of MURASHIGE and SKOOG (1962), excluding glycine, containing 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo-*

inositol, 2 mg l^{-1} BA and 1 mg l^{-1} NAA. Fifteen flasks were inoculated as described. Five were placed on a shaker under a continuous low light intensity (0.1 μmol m^{-2} s^{-1}), five were covered in foil and so kept in the dark, and the remaining five were placed under a continuous light intensity of 37 μmol m^{-2} s^{-1}. Following the transfer to sidearm flasks four weeks after initiation of the cultures, a Day 0 SCV value was determined and thereafter growth was monitored on a daily basis. After 14 days, no growth had yet occurred in any of the flasks. Aliquots of the samples were removed and examined microscopically. No signs of fungal or bacterial contamination were evident. The viability of the cultures was then determined with FDA. This showed that the cultures were dead, with only the very occasional cell still fluorescing. After two further attempts at initiating cultures on this medium, with the same lack of success, it was concluded that the callus growth medium was not suitable for the initiation of suspension cultures of *S. mauritianum*.

STREET (1977) acknowledged that it may sometimes be necessary to use a different medium for suspension culture growth than has been developed for the satisfactory culture of the primary tissue culture. It therefore seemed that initiation of suspension cultures of *S. mauritianum* was going to prove more difficult than the initiation of callus cultures, which had occurred very readily (Chapter 5). As indicated by COHEN (1986), it is simply not possible to predict success in the establishment of cultures. Various modifications of the medium were subsequently tested.

6.3.2. Substitution of 2,4-D for BA and NAA:

Table 22 revealed that 2,4-D was the hormone most frequently used in the media of *Solanum* suspension cultures and that it was frequently used exclusively. BA and NAA were therefore substituted by 2 mg l^{-1} 2,4-D. The callus was thus transferred directly from a solid MURASHIGE and SKOOG (1962) medium containing BA and NAA, to a liquid medium with 2,4-D. In all other respects, the media were identical. The same range of light conditions employed in the first trial were used and the same initiation procedure followed. After having monitored growth for 2 weeks, again no growth was obvious. Upon determining viability of the cultures with FDA, slightly more cells seemed to be viable than when BA and NAA were used as the hormone complement, but the percentage viability was still below 5 %. This medium was clearly also not suitable for the growth of *S. mauritianum* suspensions, although use of 2,4-D appeared to be preferable to the addition of BA and NAA. Therefore, despite the fact that the friable callus broke up readily to form a suspension of single cells and small cell aggregates, no growth was occurring and further alterations to the medium were necessary.

6.3.3. Use of different strengths of MURASHIGE and SKOOG (1962) medium:

The effect of reducing the strength of the growth medium was examined. MURASHIGE and SKOOG (1962) salts and vitamins were added at 1, 0.5, 0.25 or 0.125 the usual concentration. Again, 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol and 2 mg l^{-1} 2,4-D were added. The 12 flasks (three per treatment) were placed in a Gallenkamp orbital incubator under controlled conditions (see Materials and

Methods). After two weeks of monitoring growth, during which time no change in SCV was observed, samples were combined and a 55 % inoculum was transferred to fresh medium and growth monitored for a further two weeks. The results are shown in Figure 31.

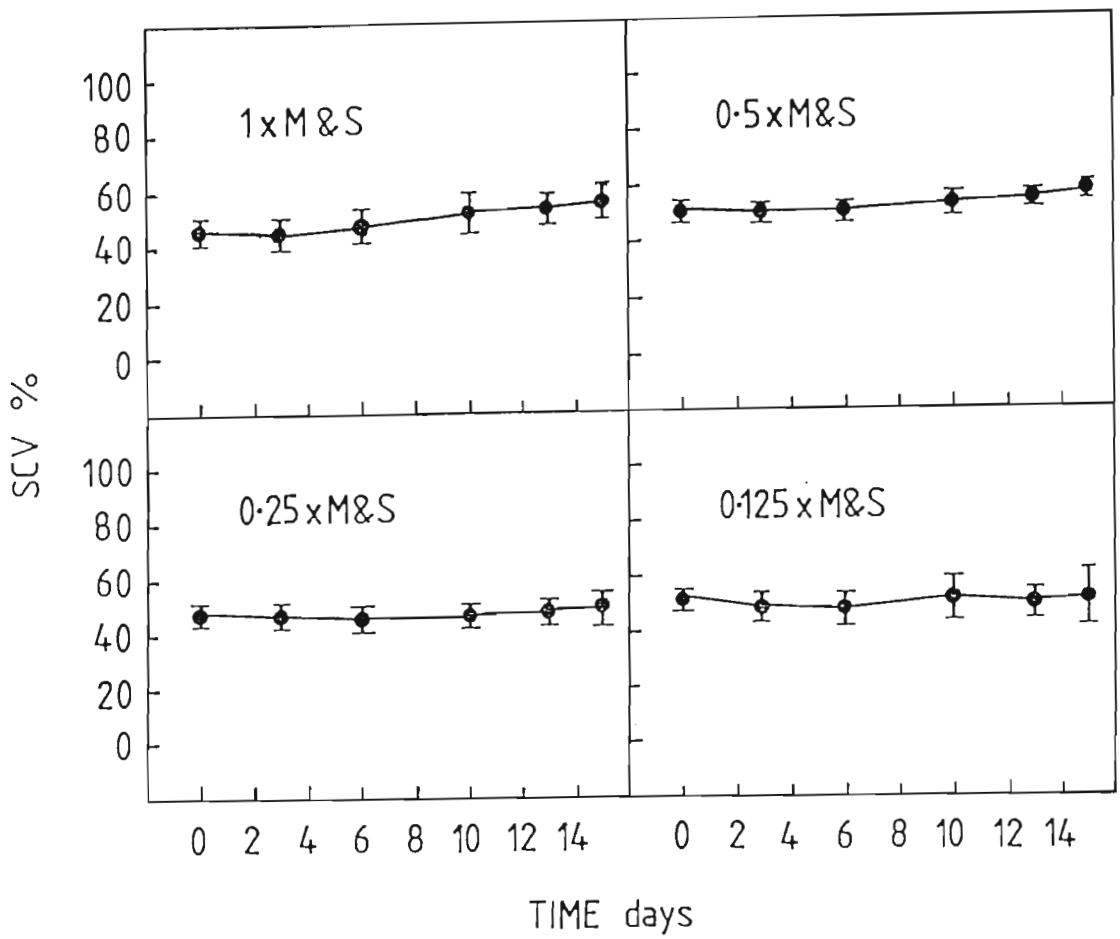

Figure 31. Growth curves of *S. mauritianum* suspensions in various strengths of MURASHIGE and SKOOG (1962) medium containing 30 g l^{-1} sucrose, 0.1 g l^{-1} *myo*-inositol and 2 mg l^{-1} 2,4-D. The vertical bars represent the standard errors.

Even with the very large inoculation volumes used in this trial, no significant growth was evident in any of the treatments. The curves certainly did not resemble typical growth curves of suspension cultures with lag, exponential growth and stationary phases. None of the treatments appeared to be any more favourable than

the others. The use of different strength media is therefore not the solution required to stimulate growth of *S. mauritianum* suspensions.

6.3.4. Addition of glycine:

Throughout the culture of *S. mauritianum* callus and suspensions thus far, a modified version of MURASHIGE and SKOOG's (1962) medium, excluding glycine, had been used. The effect of using the original recipe, which includes 0.2 mg l^{-1} glycine, was tested. The glycine was included in the vitamin stock solution. A full-strength medium supplemented with 2 mg l^{-1} 2,4-D was used and the five cultures were incubated in the orbital incubator. Large inoculation volumes (40 to 50 %) were again used upon transfer to the sidearm flasks. After three weeks of monitoring growth, just when the flasks were about to be discarded, some growth commenced in one of the flasks and after four weeks, the sidearm was almost full.

The growth curve of this culture is presented in Figure 32. Microscopic observations did not reveal the presence of any contaminants. None of the other cultures showed any signs of growth at all. This one culture had an extremely long lag phase (21 days), indicating that the medium was probably by no means optimal.

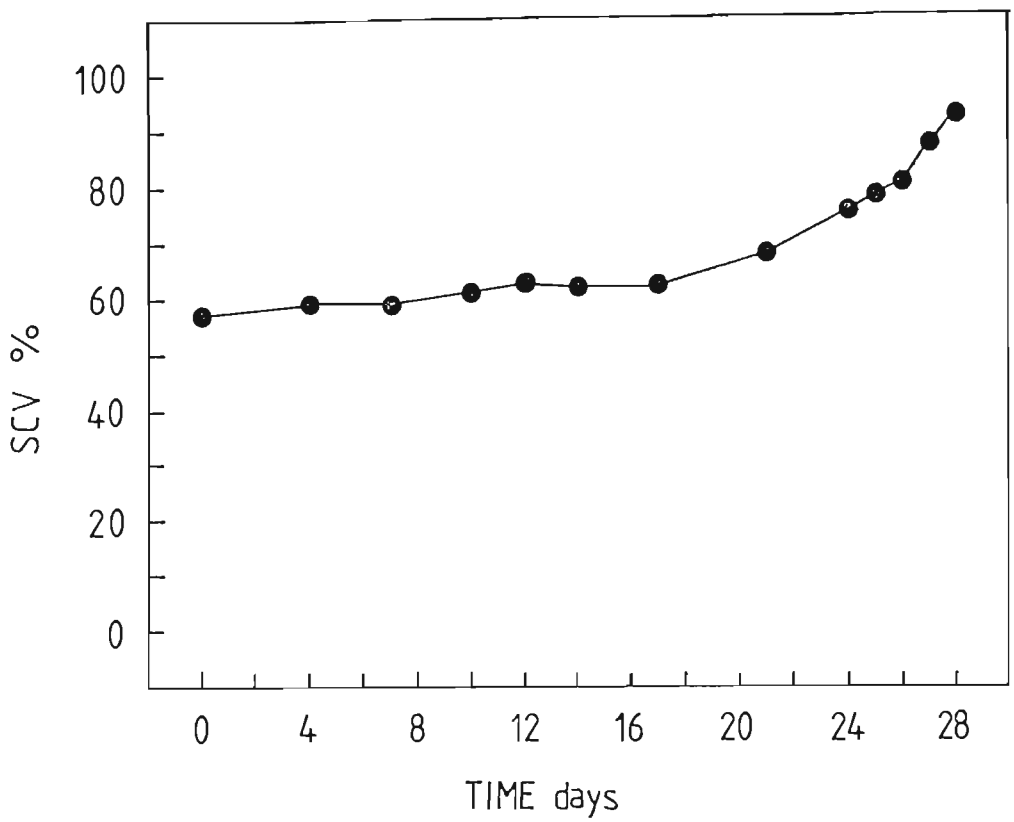

Figure 32. Growth curve of a suspension of *S. mauritianum* in original recipe MURASHIGE and SKOOG (1962) medium containing glycine and supplemented with 2mg l^{-1} 2,4-D.

The culture was subcultured into two new flasks using a 20 to 25 % inoculum. Again there was a long lag phase, but it was reduced to 14 days. After 21 days it was again subcultured, this time into six flasks with a 20 % inoculum in each. This time, by Day 5, significant growth was already observed (Fig. 33). The culture therefore appeared to be adapting to the medium.

The growth curve (Fig. 33) was now more like that expected of suspension cultures. However, the cultures had to be subcultured before entry into the stationary phase became apparent, since the cell aggregates were fairly large and the suspensions

became so thick that the sidearms of the flasks could not be emptied readily and so growth could not be determined. Viability of the cells at this stage was approximately 85 %.

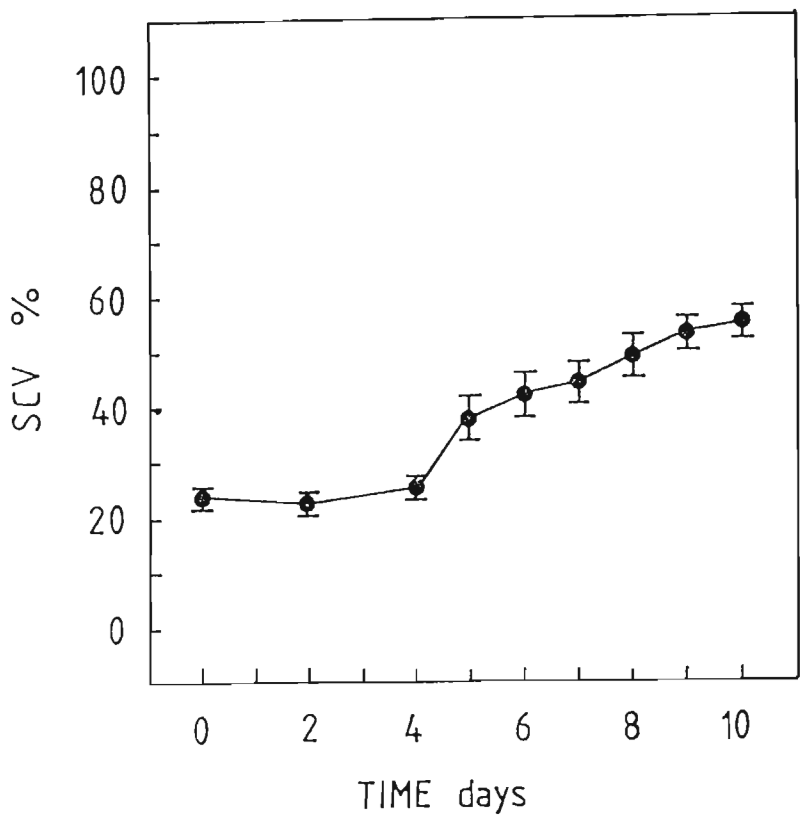

Figure 33. Growth curve after the third subculture of *S. mauritianum* cells into a MURASHIGE and SKOOG (1962) medium containing glycine and supplemented with 2 mg l^{-1} 2,4-D. The vertical bars represent the standard errors.

Finally, therefore, a viable, growing *S. mauritianum* suspension culture had been obtained. Unfortunately, at the next subculture, all the cultures were lost due to contamination as a result of inadequate sterilization of the medium and glassware. It was attempted twice more, with seven flasks at a time, to reinitiate cultures using this medium, with growth being monitored for 28 days. However, growth was

never again observed. Perhaps the bottle of callus used for this one culture was different to the others in some way and thus more amenable to forming a growing suspension on this medium. It could be concluded that this was not a reliable medium on which to initiate suspensions of *S. mauritianum*.

6.3.5. Addition of other vitamin supplements:

It has been reported (STREET, 1977) that the vitamin requirements for rapidly growing suspensions may be different to those of the corresponding callus cultures. Therefore, a trial was set up involving the following treatments:

i) MURASHIGE and SKOOG (1962) medium modified to exclude glycine with MURASHIGE and SKOOG (1962) vitamins (as control),

ii) MURASHIGE and SKOOG (1962) salts with the B5 vitamins of GAMBORG, MILLER and OJIMA (1968), and

iii) MURASHIGE and SKOOG (1962) salts with the RT vitamins of KHANNA and STABA (1968).

The composition of the three vitamin complements is given in Table 23. The B5 vitamins are very similar to those of MURASHIGE and SKOOG (1962), but far richer. The RT vitamin mixture is very different and far more complex than the others. All media were supplemented with 30 g l^{-1} sucrose and 1 mg l^{-1} 2,4-D. *Myo*-inositol (0.1 g l^{-1}) was added to the MURASHIGE and SKOOG (1962) and

vitamin stock solution. Four flasks were inoculated per treatment and the cultures subsequently all treated in the usual way.

Table 23. Composition of the MURASHIGE and SKOOG (1962), B5 and RT vitamin complements.

Vitamin	Vitamin composition (mg l^{-1} medium)		
	M & S	B5	RT
Thiamine-HCl	0.1	10	1
Niacin	0.5	1	
Pyridoxine-HCL	0.5	1	1
myo-inositol		100	
Cyanocobalamin			0.0015
Folic acid			0.5
Riboflavin			0.5
Biotin			1
Calcium pantothenate			1
Nicotinamide			2
Choline chloride			1

At the time of transfer to sidearm flasks, it was noted that two of the cultures grown in a medium containing the RT vitamins of KHANNA and STABA (1968) were far denser than any of the other cultures. Upon monitoring growth, one of these RT flasks showed definite signs of growth within three days of subculture and this continued until Day 9, when the sidearm was almost full. In none of the other treatments was any growth observed at all, even after 28 days and therefore these

treatments was any growth observed at all, even after 28 days and therefore these cultures were discarded.

The growth and secondary product synthesis in cell suspensions of another solanaceous species, *Hyoscyamus muticus* L., have also been examined in a MURASHIGE and SKOOG (1962) medium with either the original vitamin complement or with RT vitamins (KOUL, AHUJA and GREWAL, 1983). In this case, it was found that the cultures grew in both media, although those in media containing MURASHIGE and SKOOG (1962) vitamins, did tend to turn brown with time. The use of RT vitamins therefore does seem to be more favourable for solanaceous species than use of *MURASHIGE and SKOOG (1962) vitamins*. The *S. mauritianum* suspension culture in which growth had been apparent was then subcultured, but unfortunately, it became contaminated with a bacterium and had to be discarded.

It was therefore attempted to reinitiate the cultures on an RT medium. Again this was successful in several flasks (three of seven) and these were successfully subcultured and maintained in this medium. Thus, the inclusion of RT vitamins in the MURASHIGE and SKOOG (1962) medium seemed to be able to result in viable, growing suspension cultures reasonably consistently. The vitamin complement of the medium therefore appears to be very important in the culture of *S. mauritianum* cells. In contrast, in their review of the nutritional requirements of callus and cell cultures, OJIMA and OHIRA (1978) indicated mineral elements, sucrose, hormones and thiamine as indispensable, while the organic supplements, such as the other vitamins, were regarded as beneficial, but not ordinarily required.

The RT cultures were subsequently repeatedly subcultured and bulked up. A stable suspension culture of *S. mauritianum* was thus obtained. If a 25 % inoculum was used, the cultures grew and filled the sidearms in seven to eight days (Fig. 34). No significant lag phase was present under these conditions.

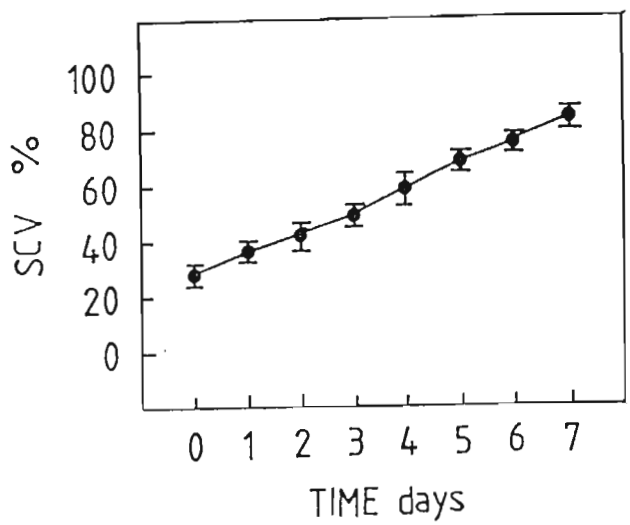

Figure 34. Growth curves of *S. mauritianum* cell suspension cultures initiated and maintained in a modified MURASHIGE and SKOOG (1962) medium supplemented with RT vitamins. The vertical bars represent the standard errors.

Growth of the cultures was very synchronous, as indicated by the small standard error bars. This growth rate of *S. mauritianum* cells is rapid compared to that of many other *Solanum* suspensions, the cultures requiring frequent subculture. In general, the occurrence and duration of each phase of the growth cycle depend on the cell type (species and strain), the frequency of subculture, the initial density and the culture medium employed (KING and STREET, 1977). In *S. jasminoides* Paxt. (JAIN and SAHOO, 1981a) and *S. xanthocarpum* Schrad. et Wendl. (KHANNA, UDDIN, SHARMA, MANOT and RATHORE, 1976), the suspensions were

subcultured at approximately six week intervals. No indication of inoculation volume was given, but overall, whatever the initial inoculum size, the growth rate appears to be considerably lower than that observed for *S. mauritianum*. Other *Solanum* suspensions, however, also have rapid growth rates. For example, suspensions of *S. eleagnifolium* Cav. were subcultured every 15 days, using a 20 % pipettable inoculum (ALVAREZ, NIGRA and GIULIETTI, 1993). For *S. mauritianum*, even if the inoculum was reduced to 10 %, the cultures had to be subcultured after nine to ten days, by which time the sidearms were full and the cultures started to become dark brown. Since such difficulty had been experienced in finding a suitable medium for initiation and growth of these cultures, the inoculation volume was not reduced further, since the smaller the inoculum, the more exacting are its growth requirements (STREET, 1977). Under the present conditions, maintenance of these cultures was very intensive.

Now that a stable *S. mauritianum* cell suspension had finally been obtained, attention could be focused on its solasodine content and the manipulation of the cultures to produce increased amounts of solasodine. In preparation for the solasodine determinations, several flasks were harvested and their fresh and dry weights measured. This gave an indication of the number of flasks that would ultimately need to be harvested in order to obtain at least 2.5 g of freeze-dried cells for extraction. The filtered cells were found to have a water content of 95.75 % and it was calculated that three flasks would provide sufficient biomass for extraction. Therefore, for subsequent trials, six flasks were cultured, to allow for contamination of some and also to provide sufficient inoculum for subculturing purposes.

6.4. Manipulation of suspension cultures: Results and Discussion

The factors that had previously been found to affect solasodine levels in *S. mauritianum* callus cultures were borne in mind when deciding on which variables to alter in order to optimize solasodine production in the suspension cultures. Three different media were ultimately tested, namely:

i) the basic MURASHIGE and SKOOG (1962) salts with RT vitamins, on which the suspensions had been initiated (MS-RT medium),

ii) the same medium, but with 3 % glucose replacing the 3 % sucrose, and

iii) half-strength MURASHIGE and SKOOG (1962) salts with full-strength RT vitamins and 3 % sucrose.

All cultures were supplemented with 0.1 g l^{-1} *myo*-inositol and 1 mg l^{-1} 2,4-D. Since the hormone complement was now totally different to that used for the callus cultures, this component of the medium was not altered at this stage.

The 3 % glucose and half-strength MURASHIGE and SKOOG (1962) cultures were initiated from cells grown in the basic MS-RT medium. The cells were poured into a measuring cylinder and allowed to settle for 10 min. The spent medium was then decanted and 20 % inocula made up to volume using the new media. The cells appeared to adapt very easily to the alterations in the media, with visible growth occurring after one day and the sidearms almost being filled after eight days. The

same basic growth pattern was maintained through the next two generations (Fig. 35).

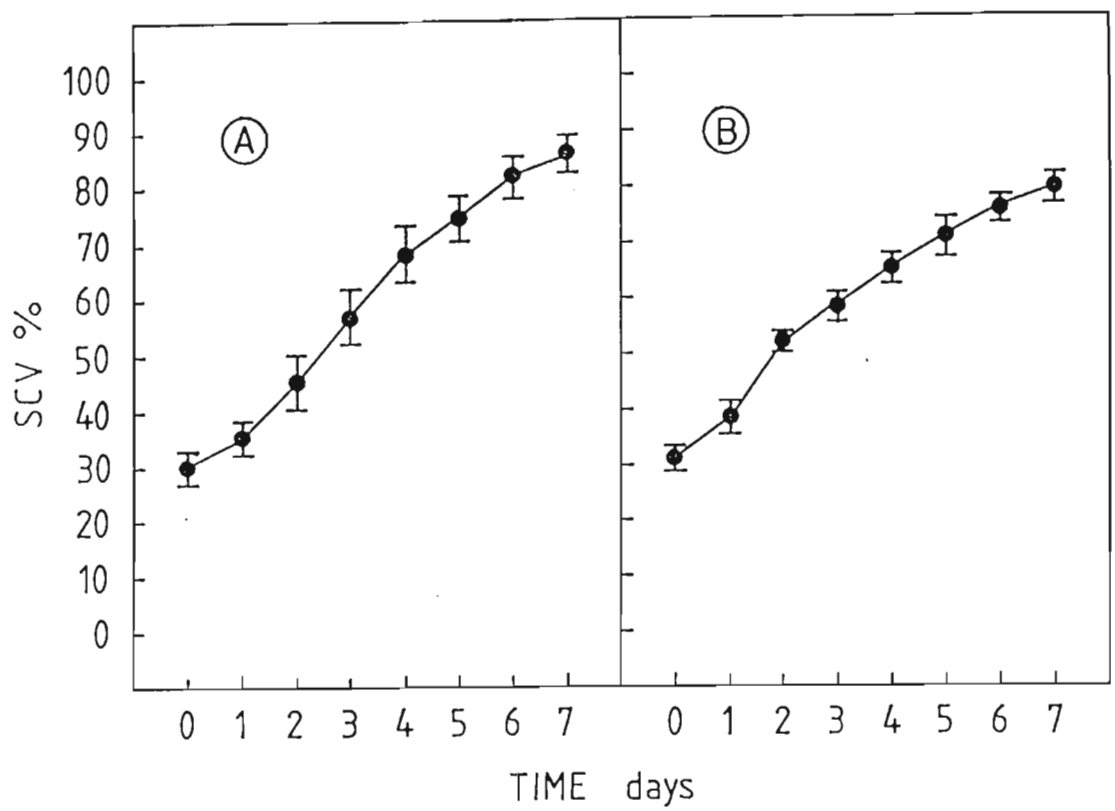

Figure 35. Growth curves of *S. mauritianum* suspension cultures grown in (A) an MS-RT medium containing 3 % glucose or (B) in a half-strength MURASHIGE and SKOOG (1962) medium containing full-strength RT vitamins and 3 % sucrose. The vertical bars represent the standard errors.

It was observed that although the growth curves of the two cultures were very similar, the suspension grown in the half-strength medium appeared to be much finer than that grown with 3 % glucose. The cells did not appear to settle as compactly over a 10 minute period. Therefore, although cell volumes were comparable, biomass was probably not. As a result, the sidearms were much easier to empty.

The cultures on all three different media were then bulked up in preparation for extraction and determination of solasodine. The cells were harvested on Day 7, using the procedure outlined in the materials and methods, just as they were entering the stationary phase. The cells were harvested at this phase, when growth is starting to decrease, because of the frequently observed negative correlation between cell growth and secondary product synthesis. There are reports, however, of solasodine production reaching a maximum with maximum growth, for example in *S. eleagnifolium* Cav. (ALVAREZ, NIGRA and GIULIETTI, 1993). As expected, the cell biomass obtained from the very fine half-strength MURASHIGE and SKOOG (1962) with RT vitamins culture was far less than that of the other two cultures (approximately half). There was, however, still more than adequate biomass for extraction purposes. The spent medium from three flasks of each treatment was also extracted and purified as described.

HPLC fractionation of the cell suspension and medium extracts yielded very clear traces (Fig. 36), but in none of them was any solasodine detected. It was therefore apparent that the solasodine-producing ability of the callus cells, which was maintained through serial subculture (Chapter 5.4.7), was lost when the cells were grown as a suspension, in the media tested. FOWLER (1985), in his review on the possibility of commercial production of secondary products *in vitro*, indicated that there are still many examples of cell suspensions that, while exhibiting good growth and high viability, do not synthesize the desired products. It seems that *S. mauritianum* falls into this category.

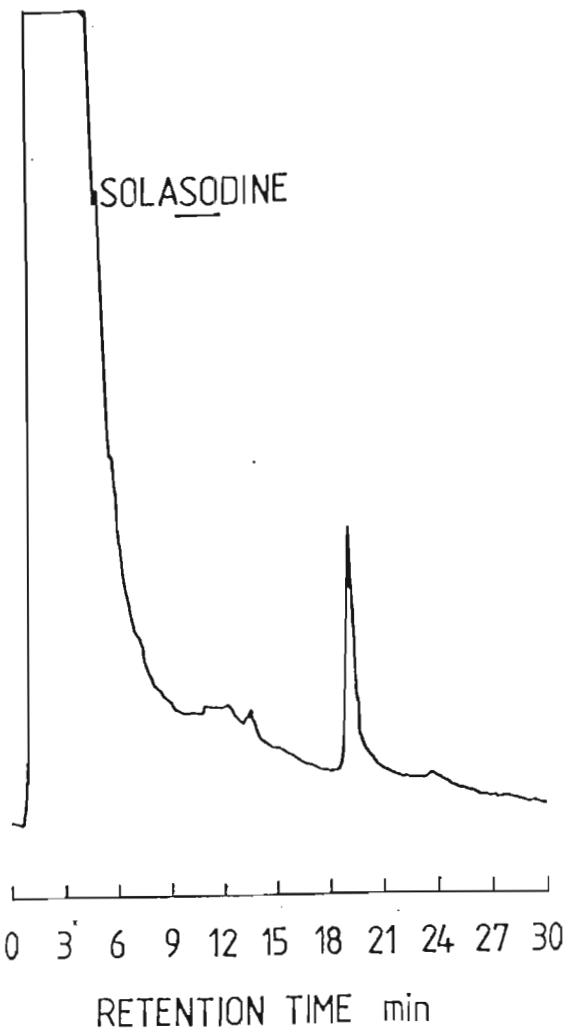

Figure 36. HPLC trace obtained following extraction and purification of a *S. mauritianum* suspension culture grown in a MURASHIGE and SKOOG (1962) salt medium with RT vitamins and 3 % sucrose. The retention time of the major peak of authentic benzoylated solasodine is shown.

It has frequently been reported in other species that callus cultures can produce higher levels of secondary products than their corresponding suspensions. Examples include *Coscinium fenestratum* L. (NAIR, SUDHAKARAN, RAO and RAMAKRISHNA, 1992), *Digitalis thapsi* L. (CORCHETE, SANCHEZ, CACHO, MORAN and FERNANDEZ-TARRAGO, 1990) and cocoa (GURNEY, EVANS and ROBINSON, 1992). However, generally, at least some level of secondary product synthesis is maintained in the suspension, unlike the situation found in *S.*

mauritianum.

Overall, therefore, although suspensions are generally regarded as the most convenient culture types for the *in vitro* production of secondary products, since they can easily be grown in bioreactors, this is not a viable option in the case of *S. mauritianum*. Perhaps further manipulation of the medium, or the addition of elicitors or precursors might have resulted in the production of some solasodine. This would, however, probably not be very high, considering that no solasodine was detectable at all in the cultures examined. If such totally negative results have been obtained for suspensions of any other *Solanum* species, they have, naturally enough, not been reported.

KURZ (1986) cited three reasons for the inability to synthesize secondary products *in vitro*:

i) The loss or mutation of genes involved in a particular step(s) of the biosynthetic pathway of a metabolite.

ii) The absence of gene expressions relating to the availability of precursors, enzymes, or the formation of cellular structures.

iii) The unavailability of substrate(s) to enzyme(s) due to local separation.

Of these, the lack of specific enzymes is apparently most frequently responsible for the inability of cell cultures to synthesize secondary metabolites.

6.5. Conclusions

Despite the ease with which *S. mauritianum* callus cultures were initiated, it proved difficult to initiate suspension cultures. The callus could not simply be transferred to the same medium lacking agar and shaken. Although the callus was friable and broke up readily when shaken, conditions were obviously not suitable for growth and ultimately the cells lost viability. After the testing of many different media, one was finally found on which cultures could be initiated with at least some consistency. This was a basic MURASHIGE and SKOOG (1962) salt medium with the RT vitamin supplement of KHANNA and STABA (1968). Once initiated, the cultures grew very rapidly on both the original initiating medium and modifications thereof. However, in none of these cultures was any trace of solasodine detectable. It can therefore be concluded that these suspension cultures of *S. mauritianum* are not suitable for the *in vitro* production of solasodine.

CHAPTER 7

HAIRY ROOT CULTURES OF *SOLANUM MAURITIANUM*

7.1. Introduction

Solanaceous species were amongst the first plants to be deliberately infected with *Agrobacterium rhizogenes* and since then, it is this family in which transformation with this bacterium has been most widely successful. Indeed, entire papers have been written on the production of hairy roots in a range of members of the Solanaceae (FLORES and FILNER, 1985; KNOPP, STRAUSS and WEHRLI, 1988; WALTON, ROBINS, BELSHAW, PARR, HAMILL and RHODES, 1988). The motivation behind many of these attempts was the possibility of producing alkaloids *in vitro*.

The genera most commonly examined have been *Atropa belladonna* L. (KAMADA, OKAMURA, SATAKE, HARADA and SHIMOMURA, 1986; ONDRĚJ and PROTIVA, 1987; SHARP and DORAN, 1990), *Scopolia* (MANO, NABESHIMA, MATSUI and OHKAWA, 1986; YIN-LIN, 1988), *Datura* (PAYNE, HAMILL, ROBINS and RHODES, 1987; OHKAWA, KAMADA, SUDO and HARADA, 1989), *Nicotiana* (HAMILL, PARR, ROBINS and RHODES, 1986; CHRIQUI, DAVID and ADAMS, 1988) and especially *Hyoscyamus* (DOERK, WITTE and ALFERMANN, 1991; OKSMAN-CALDENTEY, KIVELÄ and HILTUNEN, 1991; SAUERWEIN and SHIMOMURA, 1991; CHRISTEN, AOKI and SHIMOMURA, 1992). This plant family therefore seems highly susceptible to *A.*

rhizogenes infection. There are, however, relatively few reports of the production of hairy roots by *Solanum* species. The species that have been transformed are the potato, *S. tuberosum* L. (OOMS, BOSSEN, BURREL and KARP, 1986; VISSER, JACOBSEN, WITHOLT and FEENSTRA, 1989; OTTAVIANI, SCHEL and HÄNISCH TEN CATE, 1990) and *S. nigrum* L. (WEI, KAMADA and HARADA, 1986; MUGNIER, 1988). No reports were found of *Solanum* species in which hairy root cultures had been examined as a means of solasodine production.

There are many examples of hairy root cultures that produce levels of secondary products greater than or equal to those of untransformed root cultures or the intact plant. For example, in hairy root cultures of *Duboisia leichhardtii* F. Muell., a solanaceous species, the scopolamine content was twice that of leaves of the intact plant (MANO, OHKAWA and YAMADA, 1989). Hairy roots of *Valeriana officinalis* L. yielded valepotriates at four times the level found in the roots of nine-month-old non-transformed plants (GRÄNICHER, CHRISTEN and KAPÉTANIDIS, 1992), while one transformed line of *Chaenactis douglasii* (Hook.) H & A produced double the amount of thiarubrine of a non-transformed root culture (CONSTABEL and TOWERS, 1988). Frequently, medium composition and growth conditions have been manipulated in order to maximize production of the secondary product of interest.

Successful stimulation of secondary product synthesis is, however, not always attained. For example, in *Amsonia elliptica* Roem. et Schult., hairy roots produced the same range of indole alkaloids as normal roots and the mother plant, but at lower levels (SAUERWEIN, ISHIMARU and SHIMOMURA, 1991). The rapid

growth of hairy root cultures, however, and their frequently-observed elevated levels of secondary products suggest that the production of hairy roots is a worthwhile option to examine for the *in vitro* synthesis of secondary products such as solasodine.

Overall, therefore, it seemed likely that *S. mauritianum* would be able to be transformed by *A. rhizogenes*, since the family appears to be susceptible to this organism, although there is relatively little evidence that the actual genus is susceptible, with only a few other *Solanum* species having been examined. Solasodine, being an alkaloid, is presumably synthesized in the roots of the plant. For this reason, use of a root culture, transformed or otherwise, for the *in vitro* synthesis of solasodine, is a logical avenue to explore. In addition, differentiated cultures have frequently been observed to produce higher levels of secondary products than non-differentiated ones (Chapter 4.3).

7.2. Materials and Methods

Agrobacterium rhizogenes LBA 9402 and subsequently five more strains, namely R1600 Nester, R1601 Nester, TR 8/3 Wits, A4 Tempé and HR1 Delbert, were kindly donated by Mrs Sharmane Macrae (Forest Science and Technology, CSIR). The cultures were kept at 10°C and streaked out onto fresh medium every three weeks. Initially, the medium used was YMA (PILACINSKI and SCHMIDT, 1981; Table 24), but later, a more enriched medium, MYA (VAN WORDRAGEN, OUWERKERK and DONS, 1992; Table 24) was used. On the latter medium, production of the extracellular polysaccharide that this bacterium releases

(MORRIS, BROWNSEY, GUNNING and HARRIS, 1990) was greatly reduced.

Table 24. Media on which *Agrobacterium rhizogenes* was cultured.

Chemical	YMA	MYA
	g l^{-1}	g l^{-1}
Yeast extract	1	5
Mannitol	10	8
NaCl	0.1	5.0
$K_2HPO_4.3H_2O$	0.1	-
$MgSO_4.7H_2O$	0.2	-
Casein hydrolysate	-	0.5
$(NH_4)_2SO_4$	-	2.0
Agar	10	10
pH	7.2	6.6

For the scanning electron microscopy, leaf material of *S. mauritianum* was mounted on a copper stub. An electron microscope SP-2000 "Sputter-Cryo" low temperature system was then used for preparation of the samples. The specimens were subsequently viewed in a Hitachi 5570 scanning electron microscope and photographed.

At the time of harvest, the hairy root cultures were harvested and their fresh mass determined. They were then flash-frozen in liquid nitrogen, prior to freeze-drying. Thereafter, the samples were extracted, purified and the solasodine levels determined by HPLC, as described in Chapters 2 and 3.

7.3. Initiation of hairy roots: Results and Discussion

The way in which plants are inoculated with *A. rhizogenes* and the plant part inoculated, vary tremendously. In some instances, plants are simply inoculated *in vivo* by wounding the stems and applying the bacteria (VAN DER MARK, PIJNACKER-HORDIJK, VARGA, DE VRIES and DONS, 1990). More usually, however, inoculations are done *in vitro*. Most frequently, either leaves (CONSTABEL and TOWERS, 1988; CHRISTEN, AOKI and SHIMOMURA, 1992) or *in vitro* germinated seedlings (DAVID and TEMPÉ, 1988) are inoculated. Alternatively, callus (YOSHIKAWA and FURUYA, 1987), root discs (TEPFER and TEMPÉ, 1981; TANAKA, HAYAKAWA, MANO, OHKAWA and MATSUI, 1985) or *in vitro* grown shoots (DENO, YAMAGATA, EMOTO, YOSHIOKA, YAMADA and FUJITA, 1987) may be used. In general, if transformation has occurred, hairy roots will appear at the site of inoculation two to six weeks after infection. Thereafter, they can be removed and grown on a phytohormone-free medium. The bacterium is eliminated by the addition of antibiotics to the medium. Various different techniques were subsequently tested in attempts to induce the formation of hairy roots in *S. mauritianum*.

A range of different explant types was used, since it has been found in certain species, for example *Cucurbita pepo* L. (KATAVIĆ, JELASKA, BAKRAN-PETRICIOLI and DAVID, 1991) and *Coreopsis tinctoria* Nuttal (REICHLING and THRON, 1990), that not all parts of the same plant are equally susceptible to *A. rhizogenes*.

7.3.1. <u>In vivo</u> inoculation:

S. mauritianum plants had been grown from seed in pots and at the time of inoculation, were about 1.5 m in height. The stems of these plants were inoculated with a 24, 48 or 72 hour culture of *A. rhizogenes* LBA 9402. These cultures and all subsequent ones used for any inoculations, were grown at 25 ± 2°C. Inoculation was effected by slashing the stems with a scalpel blade loaded with *A. rhizogenes* from the plate cultures. Five plants were inoculated per treatment. In addition, a control set of plants was slashed with a sterile scalpel blade not loaded with bacteria. No roots ever developed at the site of infection. Perhaps the wounding site should have been covered, to delay drying out of the wounded area. Such *in vivo* inoculations are usually conducted with a view to improving rooting of desirable clones (ERICKSON, CELLO, FROEHLICH and BAHR, 1990; VAN DER MARK, PIJNACKER-HORDIJK, VARGA, DE VRIES and DONS, 1990) and not the production of transformed root cultures. If the transformation had been successful, the roots would then have had to be sterilized, prior to being placed in culture, which is not very practicable. This avenue was therefore not explored further.

7.3.2. Inoculation of callus:

This is not an option that is chosen very frequently, but it has been successful in *Panax ginseng* C.A. Meyer (YOSHIKAWA and FURUYA, 1987). Since abundant *S. mauritianum* callus was available, however, transformation in such a system was attempted. The callus was grown on a MURASHIGE and SKOOG (1962) medium

(excluding glycine), supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol, 2 mg l^{-1} BA and 1 mg l^{-1} NAA. Three inoculation procedures were followed:

i) The callus pieces were stabbed with a needle inoculated with *A. rhizogenes* LBA 9402.

ii) Pieces of callus were wiped over colonies of *A. rhizogenes* LBA 9402 and placed, inverted, on the medium.

iii) Loopfuls of the bacteria were dispersed in 1 ml sterile, distilled water and the resultant suspensions then poured into jars containing freshly-subcultured pieces of callus.

A control set of cultures was included, consisting of uninoculated, freshly-subcultured callus. In none of the treatments was any root production observed, even after three months. In a few samples, the bacteria had totally overrun the callus, in which case no response was expected, but this was usually not the case. Perhaps pretreatment of the callus with cellulase, as was done by YOSHIKAWA and FURUYA (1987) with *Panax ginseng*, might have made the cells more susceptible to infection by *A. rhizogenes* LBA 9402.

7.3.3. Inoculation of leaves:

The use of this more classic inoculation explant was attempted. The leaves of *S. mauritianum* were picked from trees in the Muswell Hill region of Pietermaritzburg. Young (less than 10 cm in length) leaves were used, since they were liable to be more easily sterilized than the very large, mature leaves. Also, because of their

small size, they were easier to handle. A major problem was encountered with the sterilization of the leaves. During the leaf inoculation trials, several different sterilization procedures were attempted. These included a soak in:

i) 1 % sodium hypochlorite for 20 minutes,
ii) 2.3 % sodium hypochlorite for 20 minutes,
iii) 3.5 % sodium hypochlorite for 15 minutes, or
iv) 0.1 % mercuric chloride for ten to 15 minutes.

In all cases, the leaves were initially rinsed under running tap water prior to being briefly sprayed with ethanol. All sterilants contained the surfactant Tween 20. Following sterilization, the leaves were rinsed thoroughly with four washes of sterile, distilled water. With none of the treatments was sterilization satisfactory. Contamination was frequently in the region of 50 to 60 %. The use of 3.5 % sodium hypochlorite resulted in the most consistent degree of sterilization, but leaves tended to start to bleach and become very soft. A scanning electron microscopic examination of the leaf surface revealed why the leaves were so difficult to sterilize (Fig. 37). Both leaf surfaces were covered by a dense mat of interlocking stellate hairs. This mat was so dense, that the leaf surface was not visible amongst the hairs. It was not surprising, therefore, that none of the sterilization treatments had been very effective. The only alternative was to ensure that sufficient leaves were inoculated to result in at least ten explants remaining sterile in any one treatment. This was the approach adopted.

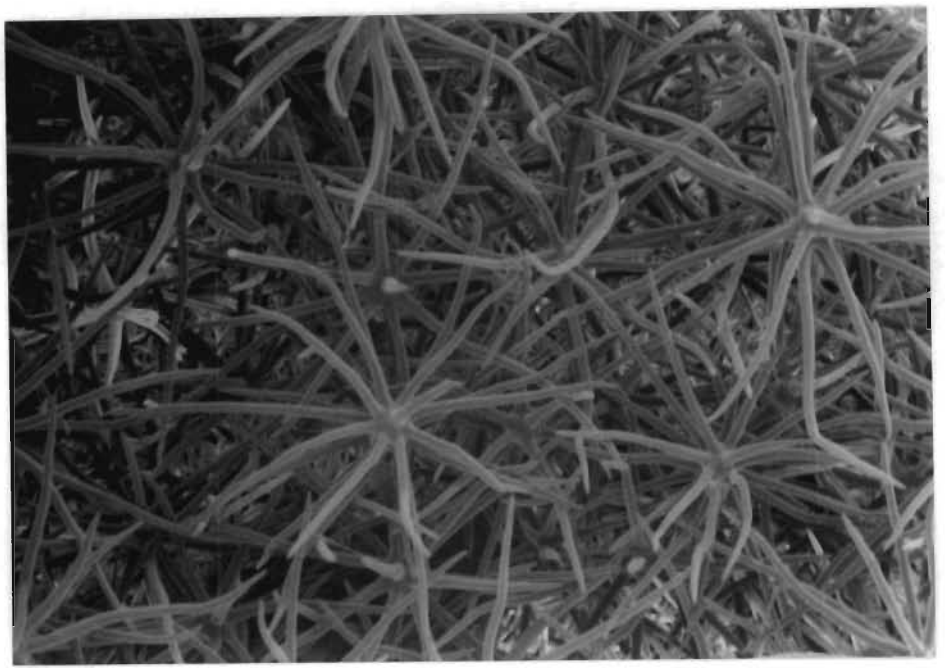

Figure 37. Scanning electron micrograph of the stellate hairs on the lower surface of *S. mauritianum* leaves (x 107).

7.3.3.3. Inoculation of leaf discs:

There are various ways in which leaves may be inoculated with *A. rhizogenes* in an attempt to induce hairy root formation. The most common method is to immerse leaf discs or segments in a bacterial suspension. The period of immersion may be as little as five minutes (MATSUMOTO and TANAKA, 1991). This is known as co-cultivation (SAUERWEIN and SHIMOMURA, 1991; CHRISTEN, AOKI and SHIMOMURA, 1992).

Plates were poured containing MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol and 2 mg l^{-1} BA and allowed to set. Leaf discs were punched from sterilized leaves using an autoclaved leaf disc puncher. Two procedures were then followed. Either:

i) The leaf discs were floated in water while loopfuls of 48 or 72 hour cultures of *A. rhizogenes* LBA 9402 were inoculated into tubes containing 1 ml sterile, distilled water each and shaken to form a suspension. These suspensions were then poured onto the surface of the agar plates. Three leaf discs were blotted onto sterile filter paper and placed onto the surface of each plate; or

ii) The leaf discs were dropped directly into a suspension made from 48 or 72 hour cultures of *A. rhizogenes* LBA 9402. After two hours, the discs were removed, blotted onto sterile filter paper to remove excess bacterial suspension, and placed on the surface of the agar in the plates.

All the plates were then sealed with parafilm and incubated at 25 $\pm$ 1 °C under a 16 hour : 8 hour photoperiod with a light intensity of 55 μmol m^{-2} s^{-1}. Observations continued for four weeks, by which time the uncontaminated leaf discs were turning brown and some of the leaf discs had become overrun by the bacterium. No root formation was observed at all.

7.3.3.2. Inoculation of leaf midribs:

An alternative to co-cultivation of leaf discs and bacteria, is the inoculation of leaf midribs (OKSMAN-CALDENTEY, KIVELÄ and HILTUNEN, 1991) or petioles (SAITOU, KAMADA and HARADA, 1991) of sterilized leaves with a scalpel or needle loaded with bacteria. It was elected to inoculate leaf midribs.

It was far easier to cut 1 cm^2 leaf segments including the midrib, than it had been to punch out leaf discs. The segments were then held firmly with forceps, while being slashed with a scalpel or pierced with a needle loaded with a 24, 48 or 72 hour culture of *A. rhizogenes* LBA 9402. The leaves were then transferred to culture tubes containing MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol and no hormones. The leaves became overrun with the bacterium in a few days. It appeared that the absence of hormones from the medium encouraged growth of the bacterium, since in the previous trials, where hormones had been added to the media, bacterial growth was far less.

Due to the problem of excessive bacterial growth, the trial was repeated, but the inoculated leaves were placed on a quarter-strength medium supplemented with only 1 % sucrose and no hormones. After five days, the explants were then transferred to a full-strength medium with 3 % sucrose, to which 250 mg l^{-1} ampicillin had been added following filter-sterilization. This transfer to antibiotic-containing medium seemed to be too late, however, since many of the explants still became swamped by the bacterium. No hairy root induction was observed in any of the explants that were not overrun with the bacterium. *S. mauritianum* was thus proving to have low susceptibility to *A. rhizogenes* LBA 9402. A new approach was therefore adopted for the infection of leaves.

7.3.3.3. Use of acetosyringone:

In nature, the infection of plants by agrobacteria is initiated by the accidental wounding of the plant. A mixture of compounds is released from the wound site and some of these compounds, at low concentration, exert a positive chemotaxis on the bacterium. It is thought that this chemotaxis is one factor that contributes to the colonization of the wound site (MIKI and IYER, 1990). One of the compounds that exerts such an effect is acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone), which is available commercially (Sigma Chemicals).

At higher concentrations, such compounds also induce the expression of several bacterial genes (MIKI and IYER, 1990). Acetosyringone is regarded as a phenolic inducer of the virulence genes of agrobacteria (FORTIN, NESTER and DION, 1992). The addition of this substance to a culture of *Agrobacterium tumefaciens* prior to incubation with leaf segments of *Arabidopsis thaliana* (L.) Heynh., increased the transformation rate from three to 63 % (SHEIKHOLESLAM and WEEKS, 1987). Similarly, the use of acetosyringone greatly promoted *A. tumefaciens*-mediated transformation in *Atropa belladonna* L., a solanaceous species (MATHEWS, BHARATHAN, LITZ, NARAYANAN, RAO and BHATIA, 1990). It was therefore attempted to increase the chances of transformation of *S. mauritianum* by *A. rhizogenes* by the addition of acetosyringone.

Loopfuls of a 48 hour culture of *A. rhizogenes* LBA 9402 were inoculated into 20 ml liquid YMA medium (Table 24) contained in 50 ml conical flasks. The flasks were stoppered with cottonwool and placed on a shaker (150 rpm) at 25 $\pm$ 1°C

under a low light intensity (0.1 μmol m^{-2} s^{-1}). After 48 hours, a small amount of dissolved acetosyringone was filter-sterilized and added to half the cultures, to make a 20 μM concentration of the phenolic. After 10 minutes, 1 cm^2 sterilized leaf segments were added to the flasks containing the *A. rhizogenes* cultures, with or without acetosyringone. The flasks were swirled intermittently. After a further 20 minutes, the leaf segments were removed with forceps and blotted on sterile filter paper to remove excess bacterial suspension. All the explants were then placed in culture tubes containing a quarter-strength medium supplemented with 1 % sucrose and 0.1 g l^{-1} *myo*-inositol and lacking phytohormones. Inositol is thought to elicit the maximum activity of acetosyringone (SONG, SHIBUYA, EBIZUKA and SANKAWA, 1990).

After three days, all the explants were transferred to a full-strength medium containing 250 mg l^{-1} ampicillin and returned to the culture room. Forty tubes were cultured for the acetosyringone treatment and another forty for the control (no acetosyringone). For no logical reason, contamination remained very low in this trial and so a clear indication of response could be obtained. The response was, however, that no hairy root induction occurred, even in the acetosyringone treatment. The acetosyringone therefore did not have the desired effect in the system tested.

Infection by *A. rhizogenes* is known to be strain-specific. For example, in *Duboisia myoporoides* R. Br., inoculation with *A. rhizogenes* strain 8196 produced no hairy roots, whereas they formed very readily following inoculation with strain HR1 (DENO, YAMAGATA, EMOTO, YOSHIOKA, YAMADA and FUJITA, 1987).

Similarly, in *Catharanthus roseus* (L.) G. Don., four strains of *A. rhizogenes* were able to initiate hairy roots, but strain TR7 was totally unable to do so (TOIVONEN, BALSEVICH and KURZ, 1989). It was thought, therefore, that the use of strains of *A. rhizogenes* other than LBA 9402, might result in successful induction of hairy roots in *S. mauritianum*.

7.3.3.4. Inoculation of leaves with additional *A. rhizogenes* strains:

Five additional strains of *A. rhizogenes* were obtained (see Materials and Methods). The midribs of 1 cm^2 sterile *S. mauritianum* leaf explants were slashed with scalpels loaded with 24, 48 or 72 hour cultures of the five additional strains. Twenty explants were inoculated per strain, for each of the three different aged cultures. After four days on a quarter-strength medium, the explants were transferred to a full-strength medium containing 250 mg l^{-1} ampicillin. By subsequently observing the degree to which the bacteria swamped the sterile leaf explants, it became obvious that the strains A4, R1601 and TR 8/3 were far more susceptible to ampicillin than the other two new strains, R1600 and HR1. With none of these strains, however, was the induction of any hairy roots observed. The entire trial was repeated, with the same outcome.

The effect of using acetosyringone was then determined. The procedure was as outlined for the previous acetosyringone trial in which only strain LBA 9402 was used (Section 7.3.3.3), except that the concentration of ampicillin in the antibiotic-containing medium was increased to 300 mg l^{-1}. Again, however, no hairy root induction was observed. In addition, the increase in ampicillin concentration had

no effect in further decreasing growth of the bacteria. The continuing frustration caused by the difficulty in sterilizing the *S. mauritianum* leaves efficiently, suggested that an alternative explant be sought for inoculation. If the levels of contamination were reduced, the number of clean explants in which to observe possible responses, would be greatly increased. Since levels of transformation may be in the region of less than one in thirty (MANO, OHKAWA and YAMADA, 1989), this is an important criterion.

7.3.4. Inoculation of hypocotyls:

It was decided that the easiest way of obtaining sterile explants for inoculation with *A. rhizogenes*, would be to raise sterile seedlings *in vitro* from seed. This approach is used frequently, for example in the inoculation of cauliflower (DAVID and TEMPÉ, 1988), *Valeriana officinalis* L. (GRÄNICHER, CHRISTEN and KAPÉTANIDIS, 1992) and *Psoralea* species (NGUYEN, BOURGAUD, FORLOT and GUCKERT, 1992). A disadvantage, is that seedlings of the appropriate age are not instantly available at all times, as leaves are, since the seeds must first be germinated.

CAMPBELL and VAN STADEN (1983) reported two methods by which seeds of *S. mauritianum* could be successfully germinated, namely by exposure to alternating temperatures or by the addition of gibberellic acid. The latter was considered to be more practicable and so preliminary germination trials were initiated. One set of seeds was imbibed in a solution of gibberellic acid (100 mg l^{-1}) overnight in a Petri dish, while another was imbibed in distilled water. The following day, both sets

of seeds were sterilized in 3.5 % sodium hypochlorite for 15 minutes, prior to being rinsed thoroughly in sterile, distilled water. One seed was then placed per tissue culture tube, containing 10 ml of solidified MURASHIGE and SKOOG (1962) medium (excluding glycine), supplemented with 1.5 % sucrose and 0.1 g l^{-1} *myo*-inositol. The cultures were incubated at 25 $\pm$ 1°C at a low light intensity (0.1 μmol m^{-2} s^{-1}). It was thought that any residual effect of the gibberellic acid should not adversely affect any subsequent induction of hairy roots, since in *Datura innoxia* Mill., gibberellic acid has been found to actually enhance growth of the hairy roots (OHKAWA, KAMADA, SUDO and HARADA, 1989).

It was found that the gibberellin-treated *S. mauritianum* seed indeed germinated far more rapidly than the untreated seed, but it was still ten weeks before most had germinated. Another trial was therefore conducted in which 500 mg l^{-1} gibberellic acid was used. This time, germination commenced within two weeks after imbibition. This was therefore the treatment used in all subsequent trials. No contamination of the seeds was observed during the germination period, the sterilization method used thus being effective.

Many different means of inoculating sterile seedlings have been used. These include the decapitation of the seedling and the inoculation of the cut surface (KYO, MIYAUCHI, FUJIMOTO and MAYAMA, 1990); inoculation of wounded hypocotyls or other parts of the intact seedlings (DAVID and TEMPÉ, 1988; NGUYEN, BOURGAUD, FORLOT and GUCKERT, 1992); or the inoculation of various excised portions of the seedlings (DOERK, WITTE and ALFERMANN, 1991; ISHIMARU and SHIMOMURA, 1991; OKSMAN-CALDENTEY, KIVELÄ

and HILTUNEN, 1991). It was decided to inoculate excised hypocotyls of the *S. mauritianum* seedlings. At the time of use, the seedlings were approximately 3 cm in height (four to six weeks after imbibition). Inoculation of hypocotyls has been used successfully for the induction of hairy roots in *Catharanthus roseus* (L.) G. Don. (JUNG, KWAK, KIM, LEE, CHOI and LIU, 1992) and *Cucumis sativus* L. (TRULSON, SIMPSON and SHAHIN, 1986).

All six strains of *A. rhizogenes*, including LBA 9402, were tested simultaneously, using 48 hour cultures of the bacteria. Hypocotyl segments (1.5 cm) were excised from the *S. mauritianum* seedlings and the basal ends touched onto a bacterial colony. Ten hypocotyls were inoculated per bacterial strain. The hypocotyl segments were then inverted and inserted into the medium. They were inverted to try to reduce the rapid growth of the bacterium on the medium surface, which was previously found to engulf explants. This problem of excessive bacterial growth has been reported by other researchers (BERLIN, FECKER, RÜGENHAGEN, SATOR, STRACK, WITTE and WRAY, 1991; HOSOKI, KIGO and SHIRAISHI, 1991).

The medium used was quarter-strength MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 1 % sucrose, 0.1 g l^{-1} *myo*-inositol and without hormones. The cultures were then incubated at 25 $\pm$ 1°C under a low light intensity (0.1 μmol m^{-2} s^{-1}). After five days, the hypocotyls were transferred to a full-strength medium supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol and containing 300 mg l^{-1} ampicillin. This time, the infected ends of the hypocotyls were inserted into the medium. At this stage, none of the explants had yet become

engulfed by the growth of the bacteria, due to the inversion of the hypocotyls. Thereafter, the explants were transferred to fresh antibiotic-containing medium at weekly intervals. In this way, the excessive growth of the agrobacteria was contained and gradually reduced, except with strain R1600, which appeared to be very resistant to ampicillin.

A set of controls was initiated at the same time that the hypocotyls were inoculated with the *A. rhizogenes*. It consisted of hypocotyl segments that had not been inoculated with the bacterium. In all other respects, the control hypocotyls were treated in the same way as the inoculated ones, initially being inverted and subsequently being transferred to antibiotic-containing media. After 12 days, some rooting of the hypocotyls was observed, especially in the control treatment (Table 25).

Table 25. Number of tubes (out of ten) in which rooting of hypocotyls was observed 12 days after inoculation with *A. rhizogenes*.

Agrobacterium strain	Rooting (out of ten)
None (control)	7
A4	2
R1601	5
TR 8/3	4
LBA 9402	6

The results for strain R1600 are not given, since most of the explants had now been engulfed by the bacterium. The roots were usually produced at the hypocotyl base, but some were present along the length of the hypocotyl inserted into the medium.

In no treatment did the degree of rooting at this stage appear to be any different to that observed in the control, uninoculated segments. These roots were therefore regarded as normal, untransformed roots that had formed spontaneously. With time, the number of tubes in which rooting occurred increased slightly in all treatments, but in general, the degree of rooting did not. The exception was one of the hypocotyls inoculated with strain LBA 9402. In this one tube, the number of roots increased greatly with time, began to grow in all directions and branched considerably. The difference between this tube and one of the control ones, is shown in Figure 38.

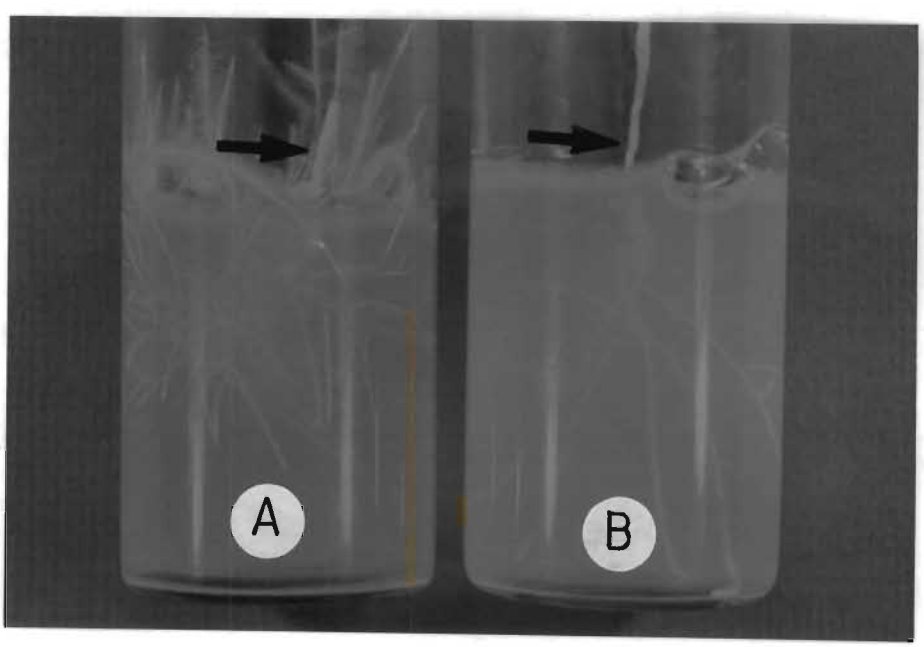

Figure 38. Roots produced from (A) a hypocotyl inoculated with *A. rhizogenes* LBA 9402 and (B) an uninoculated, control hypocotyl. The original hypocotyls are indicated by the arrows.

The appearance of this one tube was so different to that of any other tubes, that it was concluded that transformation might finally have occurred. Since one of the properties of hairy roots is that they grow vigorously in the absence of hormones (SHARP and DORAN, 1990), the roots were excised from the hypocotyl and placed on a MURASHIGE and SKOOG (1962) medium (excluding glycine) containing 3 % sucrose, 0.1 g l^{-1} *myo*-inositol, 300 mg l^{-1} ampicillin and no hormones. The medium was contained in 65 mm sterile plastic Petri dishes. Thirty roots were treated in this way. In addition, ten roots from the control hypocotyl segments were placed in Petri dishes on the same medium. All the dishes were then sealed and transferred to a culture room at 25 $\pm$ 1°C in the dark. Within days, many of the putatively transformed roots had started to grow rapidly and to branch in all directions, as would be expected of a hairy root culture. In contrast, the control roots grew very slightly and slowly and branched very little (Fig. 39).

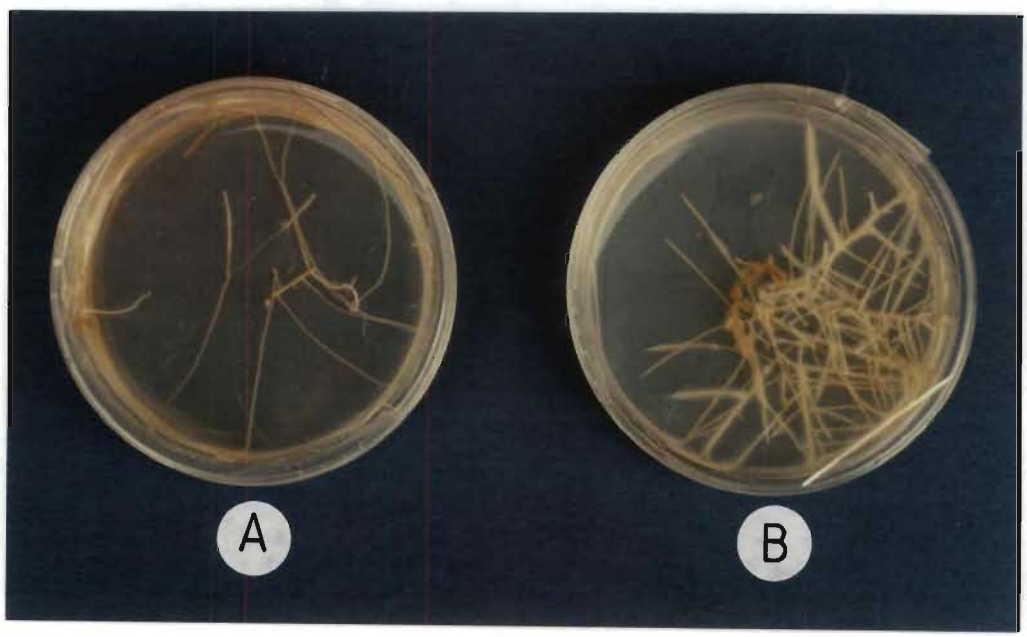

Figure 39. A comparison between the growth of (A) control roots and (B) those thought to have been transformed with *A. rhizogenes* LBA 9402 (B). The control roots were photographed six weeks after the initial subculture into Petri dishes and the "hairy" roots, two weeks after a subsequent subculture.

Overall, the roots from the hypocotyl inoculated with *A. rhizogenes* strain LBA 9402 had all the visible characteristics of a hairy root culture (see Chapter 4.5.1.3), namely diminished geotropism, a high degree of lateral branching, the ability to grow very rapidly in the absence of externally-supplied hormones and they could be subcultured, which implied that they were capable of permanent growth. It was therefore concluded, that transformed roots had indeed been induced. Other means of determining this with more certainty, would be to test for the presence of opines by paper electrophoresis and silver staining (PETIT, DAVID, DAHL, ELLIS, GUYON, CASSE-DELBART and TEMPÉ, 1983) or by Southern hybridization (NODA, TANAKA, MANO, NABESHIMA, OHKAWA and MATSUI, 1987).

For subculture, one to two centimetre segments of the hairy roots were transferred to fresh medium, with or without ampicillin. The two media were used in order to determine whether or not all the bacteria had yet been eliminated. On an antibiotic-containing medium, growth of the bacteria was sometimes suppressed and so not visible, whereas on transfer to an antibiotic-free medium, the bacteria would again spread and grow out onto the medium, if present. This monitoring process was conducted at two to three weekly intervals, by which time the Petri dishes were full of roots. After four such transfers, the bacteria had been removed from most of the cultures and the antibiotic could be eliminated from the medium.

With the amount of growth and branching occurring in the cultures, rapid bulking up of these cultures in preparation for solasodine analysis seemed feasible. From one initial tube in which transformation was successful, an almost unlimited number of roots should be able to be obtained in a short time.

It was then attempted to repeat the transformation process, using only strain LBA 9402. The cultures were grown for 24, 48 or 72 hours and then used to inoculate hypocotyls, as describe previously. Twenty-five hypocotyls were inoculated per treatment. After five days, the explants were again transferred to an antibiotic-containing medium. Again, a low degree of spontaneous rooting occurred, but in none of the tubes was the massive production of roots in all directions observed. *S. mauritianum* therefore seems to have very low susceptibility to *A. rhizogenes*, including strain LBA 9402.

In the literature, transformation frequencies are not always reported. When they are, the rate of transformation is very varied, depending on the bacterial strain and the plant species involved. For example, in *Catharanthus roseus* (L.) G. Don., strain TR7 was totally unable to induce hairy roots, while four other strains had an infection rate of 60 to 80 % (TOIVONEN, BALSEVICH and KURZ, 1989). Similarly, in *Duboisia myoporoides* R. Br., several hundred shoots were inoculated with *A. rhizogenes* strain 8196, but no roots formed, whereas with strain HR1, there was no problem with hairy root induction (DENO, YAMAGATA, EMOTO, YOSHIOKA, YAMADA and FUJITA, 1987). In a whole range of solanaceous species (*Atropa, Datura, Hyoscyamus, Scopolia*) an average of 80 % of the explants infected with strain A4 produced hairy roots (KNOPP, STRAUSS and WEHRLI, 1988). It is unusual that such a wide range of species is so successfully transformed. In the three examples mentioned, where transformation did occur, the infection rate was very high, implying that there either was a response, or none, depending on the bacterial strain used. This is not necessarily the case. In *Salix alba* L., 15 % of the shoots responded to infection with *A. rhizogenes* strain 15834

with the formation of hairy roots (HAUTH and BEIDERBECK, 1992). Such an intermediate response is frequently observed.

Perhaps if even more strains had been tested on *S. mauritianum*, one might have been found to which the species was more susceptible. In general, however, it can be concluded that its resistance to *A. rhizogenes* seems to be very high. This, therefore, is a major limiting step in any potential production of solasodine in hairy root cultures of *S. mauritianum*. As demonstrated, however, once a single event of transformation has occurred and a few hairy roots have been produced, a large number of cultures can be obtained in a short time. Naturally, if the transformation rate were higher, different lines could be established that could be screened for solasodine-producing ability. The next step was to determine whether or not the hairy root cultures of *S. mauritianum* that had been established, produced solasodine.

7.4. Manipulation of hairy root cultures: Results and Discussion

As had been the approach with the manipulation of the suspension cultures of *S. mauritianum*, the factors that had increased solasodine productivity of the callus cultures (Chapter 5) were tested to determine their effect on solasodine content of the hairy root cultures. The two factors of interest were a reduction in medium strength by half and the substitution of 3 % glucose for 3 % sucrose. The other medium component that had had an effect on solasodine content of *S. mauritianum* callus was the hormone complement. Since hairy roots grow well in the absence of phytohormones, this factor was not examined. It was then attempted to transfer

hairy roots to the two new media and to observe their growth on the altered media. The media tested were a half-strength MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 3 % sucrose and 0.1 g l^{-1} *myo*-inositol and a full-strength medium supplemented with 3 % glucose and 0.1 g l^{-1} *myo*-inositol. Root tips (1 to 2 cm) or segments of roots with protruding lateral roots were transferred onto these media and fresh medium of the original type. The dishes were sealed with parafilm and incubated in the dark at 25 $\pm$ 1°C.

It soon became apparent that the hairy roots grew very readily on the half-strength medium, but when 3 % glucose was substituted for 3 % sucrose in a full-strength medium, growth was very slow (Fig. 40).

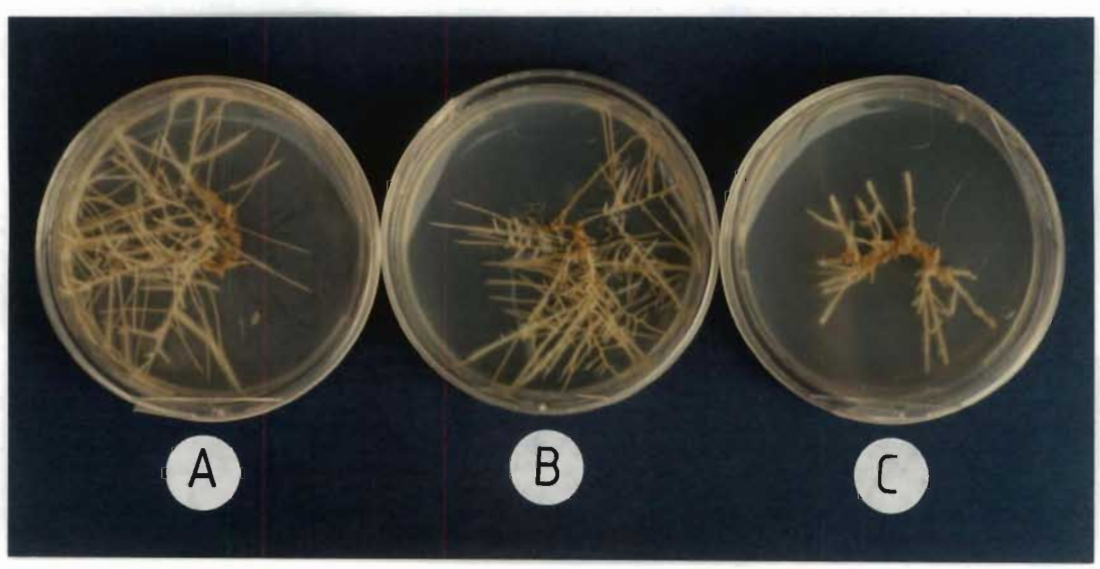

Figure 40. A comparison between the growth of hairy root cultures of *S. mauritianum* grown on (A) a full-strength MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 0.1 g l^{-1} *myo*-inositol and 3 % sucrose, (B) a half-strength medium supplemented with 3 % sucrose and (C) a full-strength medium supplemented with 3 % glucose.

In the 3 % glucose treatment, callus formation originating from the original root explant and radiating out along the roots that developed, was greater than in the other two treatments (Fig. 40). Cultures with a very similar appearance were observed to be produced by certain slow-growing hairy root clones of potato (OTTAVIANI, SCHEL and HÄNISCH TEN CATE, 1990). After three weeks, these three *S. mauritianum* culture types were subcultured onto the same media and the same growth patterns were observed. This continued through subsequent generations.

It was then attempted to grow the hairy roots in liquid culture, since this would allow for easier harvesting of the roots. On a solid medium, the roots grow all through the agar, which makes harvesting very time-consuming. Root explants (15) from each of the three different media were then transferred to 10 ml of the same liquid medium contained in 50 ml conical flasks. The flasks were securely stoppered with cottonwool. The flasks were then placed on a shaker at 100 rpm and kept at a temperature of 25 $\pm$ 2.5°C under a light intensity of 0.2 μmol m^{-2} s^{-1}. The same growth trends were observed on the three media when liquid (Fig. 41), as when solid (Fig. 40), with growth in the medium containing 3 % glucose being greatly reduced relative to that in the other two media. Therefore, unlike the transfer of *S. mauritianum* callus to a liquid medium (Chapter 6), no alteration of the medium was necessary for growth to continue in a liquid culture, since the growth pattern was unchanged. The cultures from all three treatments were subsequently bulked up (fifty flasks per treatment) and harvested in the usual way after three weeks.

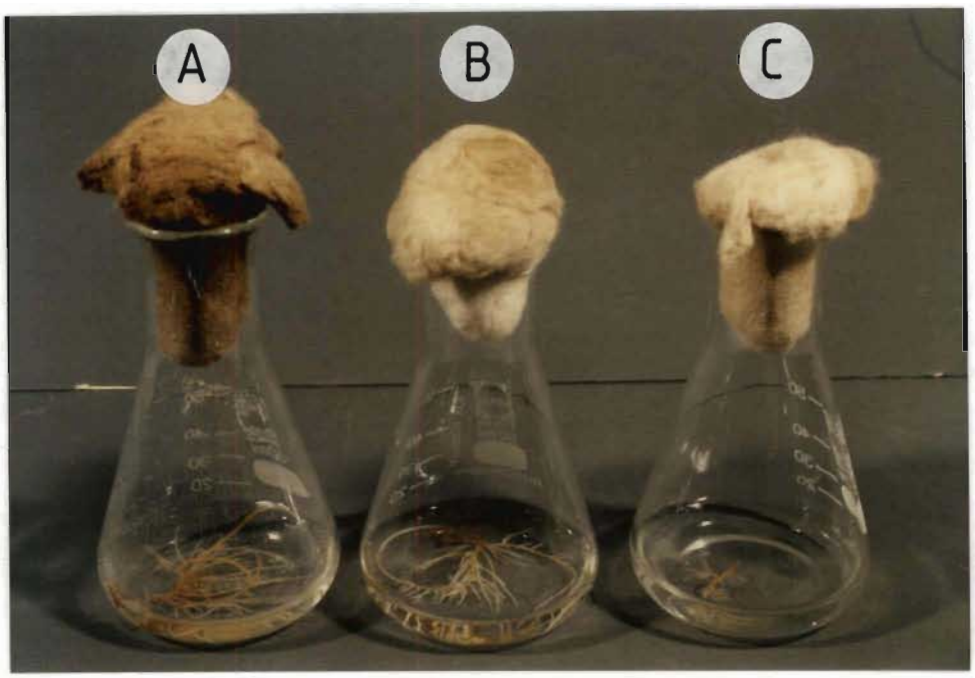

Figure 41. A comparison between the growth of hairy roots of *S. mauritianum* grown in (A) a full-strength MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 0.1 g l^{-1} *myo*-inositol and 3 % sucrose, (B) a half-strength medium supplemented with 3 % sucrose and (C) a full-strength medium supplemented with 3 % glucose.

Concurrent with the establishment of liquid hairy root cultures, it was attempted to initiate root cultures from untransformed roots, as a means of comparison. Preliminary trials indicated that root tips from *in vitro*-germinated *S. mauritianum* seedlings would grow on a MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 2 % sucrose, 0.1 g l^{-1} *myo*-inositol and 0.5 mg l^{-1} NAA. Therefore, more seedlings were germinated *in vitro*, as described earlier (Section 7.3.4). The root tips (1 to 2 cm) of six-week-old seedlings were dropped into 10 ml of liquid medium contained in a 50 ml conical flask. The cultures were incubated on a shaker (100 rpm) at a temperature of 25 $\pm$ 2.5°C and under a light

intensity of 0.2 μmol m^{-2} s^{-1}. After two weeks, many of the roots appeared to have thickened and some had protuberances. With time, the swollen roots developed into a brown callus from which, in some instances, roots were produced (Fig. 42).

Figure 42. Untransformed root cultures of *S. mauritianum* grown in a liquid MURASHIGE and SKOOG (1962) medium (excluding glycine) supplemented with 2 % sucrose, 0.1 g l^{-1} *myo*-inositol and 0.5 mg l^{-1} NAA.

The cultures did not have a very healthy appearance and so no attempts were made to subculture them. Staining of some of the sloughed-off cells in the medium with fluorescein diacetate, however, indicated that the cells were still viable. Growth was very slow and after six weeks the cultures were harvested. Only just over one gram (dry weight) of material was obtained from the fifty flasks, following flash-freezing in liquid nitrogen and freeze-drying. The samples were then stored until required for analysis.

As an additional comparison, large quantities of *S. mauritianum* seeds were germinated on filter paper in Petri dishes. The dishes were incubated at 25 ± 1°C under non-sterile conditions, following imbibition in 500 mg l^{-1} gibberellic acid. After 28 days, the roots were harvested. This proved to be a very tedious process, since each root (about 1.5 cm in length) had a fresh mass of less than one milligram. Ultimately, a total mass of 150 mg (dry weight) was harvested. The transformed and untransformed root samples were then extracted and purified, before the solasodine levels were determined by HPLC. The results are given in Table 26.

Table 26. Levels of solasodine extracted from various transformed and untransformed root samples of *S. mauritianum*.

Sample	Solasodine content ($\mu g\ g^{-1}DW$)	Growth index (± S.E.)
Seedling roots	503	-
Root culture	218	-
Hairy root culture: 1 x M&S; 3% sucrose	126	67.5 ± 4.9
Hairy root culture: 0.5 x M&S; 3% sucrose	41	36.3 ± 3.3
Hairy root culture: 1 x M&S; 3% glucose	97	4.6 ± 0.4

$$\text{Growth index} = \frac{\text{Final fresh weight - Initial fresh weight}}{\text{Initial fresh weight}}$$

The seedling roots contained extremely high levels of solasodine. In fact, this was higher than any levels previously recorded in this study, even the green berries only yielding 184 $\mu g\ g^{-1}DW$ (Chapter 3). Despite the fact that only 150 mg (dry weight)

of material was extracted, a large solasodine peak was present in the HPLC profile. Possibly, high levels of solasodine are synthesized in the young seedlings and then distributed to other parts of the plant as it grows. What function such high levels of solasodine could possibly have in the tiny seedling, is unknown. The amount of solasodine per seedling is very low, since they are so small, but perhaps the secondary product does act as a feeding deterrent for the entire population of tiny seedlings.

The untransformed root culture also yielded high levels of solasodine (Table 26). However, considering the unhealthy appearance of the cultures (Fig. 42) and the fact that this was a first generation culture initiated from high solasodine-yielding seedling roots, it is likely that much of this solasodine is residual, originating from the seedling root explants. This level of solasodine was greater than that recorded in hairy roots grown in any of the three media tested (Table 26). However, it must be borne in mind, that the hairy roots had been subcultured for several generations prior to extraction and there would therefore be no residual solasodine present from the hypocotyl from which the roots were initiated. Whether the hypocotyls originally contained high levels of solasodine, is not known, as this was not tested. This solasodine content value for the hairy roots is therefore genuinely a reflection of the synthesis of solasodine in the cultures. Of the hairy roots grown on the three different media, those on full-strength MURASHIGE and SKOOG (1962) medium with 3 % sucrose yielded both the highest solasodine content and maximum growth (Table 26). This is therefore the most productive of the hairy root cultures. This solasodine content was far higher than that previously recorded in any vegetative part of the intact plant (1 m high; Chapter 3.3.5), including the roots, and

approached that of the green berries. In addition, it was greater than that of any of the callus cultures (Chapter 5). The differentiated culture therefore had a higher solasodine content than the undifferentiated one, as is frequently the case (Chapter 4.3).

Despite the very low growth rate of the hairy roots grown in the medium containing 3 % glucose instead of 3 % sucrose, these cultures yielded relatively high levels of solasodine on a per gram basis (Table 26). In fact, this level was considerably higher than any levels recorded in the callus cultures of *S. mauritianum*. Because of their low growth rate, however, these cultures do not present a viable system for solasodine synthesis. A moderate growth rate and relatively low solasodine levels were found to be present when the hairy roots were grown in a half-strength medium with 3 % sucrose (Table 26).

Overall, therefore, the hairy roots grown in a full-strength medium with 3 % sucrose, yielded higher levels of solasodine than any other cultures examined in this study. In addition, the solasodine content of these roots was considerably greater than that of the vegetative parts of an intact plant and approached that of the green berries, in which solasodine is concentrated. Taking into account the ease with which the hairy root cultures could be grown and continually subcultured, these cultures do seem to provide a very favourable system for the *in vitro* synthesis of solasodine. Due to their very low mass and the fact that they would have to be destructively harvested, the germination of *S. mauritianum* seed and harvest of very young seedlings, is not a practicable means of producing solasodine, despite the very high solasodine content of the seedlings.

The present study illustrates the confusion that can result when attempting to relate *in vitro* levels of a secondary product to those of the intact plant. Perhaps this is why a comparison is frequently not made. The age and part of the plant extracted can greatly influence the resultant yield that is recorded. The situations in an *in vitro* system and an *in vivo* plant are not really comparable. The results from the extraction of an intact plant are, however, useful as a guideline to get an indication of whether the levels of the secondary product *in vitro* are reasonable or not. Dismissal of an *in vitro* system, simply because secondary product levels are not greater than those recorded in a particular part of an intact plant, is rather hasty.

A major question is which part of the plant is most relevant to use as a means of comparison. MANO, OHKAWA and YAMADA (1989) compared the scopolamine content of a *Duboisia leichhardtii* F. Muell. hairy root culture with that of the leaves of the plant. This plant part was possibly chosen because the hairy roots were initiated from inoculated leaf discs, or because this was the material most readily available. In contrast, the lobeline content of hairy roots of *Lobelia inflata* L., which were induced on seedling stems, was compared with that of roots of the plant cultivated in pots (YONEMITSU, SHIMOMURA, SATAKE, MOCHIDA, TANAKA, ENDO and KAJI, 1990). In *Atropa belladonna* L., the comparison was made between the atropine and scopolamine content of untransformed roots from one-year-old plants grown in the field, untransformed roots grown *in vitro* and hairy roots (KAMADA, OKAMURA, SATAKE, HARADA and SHIMOMURA, 1986). Roots are the most logical plant parts to compare with hairy roots, but obtaining these from intact plants in often a problem, since the plant must be destructively harvested. Overall, it seems best to extract various parts of the plant that are

available, preferably at different ages. The initiation of an untransformed root culture also provides a good means of comparison, but this should preferably be one that can be subcultured, unlike the one initiated from *S. mauritianum*. The level of the relevant secondary product is not the only criterion that must be taken into account when evaluating the usefulness of a particular culture system. Other important factors include the ease of culture and harvest and the growth rate. In the present study, the hairy root culture of *S. mauritianum* cultured in a full-strength MURASHIGE and SKOOG (1962) medium with 3 % sucrose grew rapidly and could be easily subcultured and harvested. It does, therefore, seem to provide a favourable system for the *in vitro* production of solasodine.

7.5. Conclusions

Solanum mauritianum appears to have a very low susceptibility to any of the *Agrobacterium rhizogenes* strains tested. One instance of transformation was obtained when the excised hypocotyl of an *in vitro*-grown seedling was inoculated with strain LBA 9402. From this, hairy root cultures were able to be established. These cultures exhibited poor geotropism, a high degree of branching and a rapid growth rate on a hormone-free medium. The hairy roots could readily be subcultured onto solid or into liquid media. The roots were found to produce solasodine in each of the three different growth media tested, although the solasodine content and root growth rate varied greatly. A full-strength MURASHIGE and SKOOG (1962) medium supplemented with 3 % sucrose gave the highest yield of solasodine and also the fastest growth rate. This level of solasodine was considerably greater than that previously recorded in the vegetative

organs of the intact plant, including the roots, but less than that of the green berries. The hairy root system seems to provide a favourable means of solasodine production *in vitro*.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

Solanum mauritianum Scop. (bugweed) is a problem plant in South Africa. As a weed, it is an efficient competitor for space, light, water and nutriment and frequently replaces the preferred, indigenous vegetation. Like other *Solanum* species, *S. mauritianum* has been found to contain the steroidal alkaloid, solasodine, in its green berries. This compound can be used as a substitute for diosgenin by the pharmaceutical industry, in the manufacture of steroids. The synthesis of steroid drugs using plant-derived raw materials is often simpler and sometimes cheaper, than manufacture by total synthesis. It has therefore been suggested (CAMPBELL and VAN STADEN, 1990), that the widespread collection of the fruit of *S. mauritianum* for the extraction of solasodine, could be used as a strategy for weed control. Research into this possibility is being conducted by the Plant Protection Research Institute (Cedara).

If this strategy were successful, however, supplies of the berries would ultimately dwindle and become scarce. Although the weed would then no longer be a problem, the pharmaceutical production system that had developed in the interim, would collapse. In order to prevent this, two options would be available. The first would be to cultivate the species, but this would lead to the risk of the plant again escaping into the wild and becoming a problem. The second option would be to produce solasodine in some controlled system. The direction of this dissertation then, has been to initiate and investigate the solasodine content of various different

culture types of *S. mauritianum*.

The production of secondary products, such as solasodine, in a controlled tissue culture system has certain advantages over the extraction of the chemicals from plants. These include a lack of seasonality in supply, no problems as a result of drought and disease and rapid biomass production. Plant tissue culture is, however, very labour intensive and therefore costly. The desired compound should therefore preferably fetch a high price. Diosgenin is one such compound, which suggests that the *in vitro* production of its nitrogen analogue, solasodine, is a viable option to explore.

The major stumbling block in such *in vitro* systems is the frequently-observed poor productivity. There are exceptions to this, however, and presumably, once we have a greater understanding of the physiological and genetic mechanisms underlying and controlling secondary product biosynthesis, the proportion of successes will increase. Unfortunately, at present, the approach must, of necessity, be rather empirical, since it cannot be predicted whether or not a particular species will produce the compound of interest *in vitro*. There are, however, many factors that have been observed to affect secondary product synthesis *in vitro* in some way. Although the underlying reasons for the effects are not, in general, known, such observations can be used as a guideline in the manipulation of cultures to produce elevated levels of the desired secondary product. This was the approach adopted in this study.

Callus cultures were readily initiated from the green berries of *S. mauritianum*. Although callus cultures are not generally economically viable systems for secondary product synthesis, since they cannot be grown in a conventional bioreactor, they are an essential stepping-stone to suspension cultures. In addition, in the present study, the callus cultures were used as a test system, to determine alteration of which factors would affect solasodine levels *in vitro*. It was hoped that this could then be extrapolated to suspension and other culture systems of the same species. Factors that did not affect solasodine content included the substitution of Gelrite for agar as the gelling agent, alteration of the carbon : nitrogen ratio and a reduction in the level of the micronutrients. Only one of the factors examined actually decreased solasodine content of the callus, namely the substitution of fructose for sucrose as the carbohydrate source. Many factors were found to increase solasodine content of the callus, but some simultaneously decreased callus growth to such an extent, that no advantage was gained by the manipulation. An example is the limitation of phosphate. Factors which significantly increased the overall solasodine productivity of the callus were the substitution of glucose for sucrose, the use of certain hormone combinations and a reduction in the medium strength by half. On a per gram basis, these levels of solasodine were comparable to those of the vegetative parts of an intact *S. mauritianum* plant, but approximately three times lower than those of the green berries. Overall, therefore, a good indication was obtained of the factors that increased solasodine content of the callus.

From the callus cultures, suspension cultures were initiated. Despite the lack of differentiation of such cultures, which has often been observed to result in low levels of secondary product synthesis, suspensions lend themselves to growth under

bioreactor conditions and are therefore frequently the system of choice when attempting to produce secondary products in culture. Using the factors that had increased solasodine productivity of the callus cultures as a guideline, the suspensions were grown in a variety of different media. The extrapolation from the callus system to the suspension culture system was not valid with respect to solasodine production, however, as no trace of solasodine was detected in any of the suspension cultures. Relative growth of the cultures on the different media did follow the same general trend.

As a contrast, a differentiated culture was tested, namely a hairy root culture. This was initiated by infecting sterile *S. mauritianum* hypocotyls with *Agrobacterium rhizogenes*. Such hairy root cultures have been successfully grown in modified bioreactor systems. Again, the factors that had been found to increase solasodine productivity of the callus cultures, were manipulated. This time, the patterns of growth did not follow those of the callus cultures, possibly as a result of the altered genetic makeup of the roots. In all cases, however, solasodine was produced in the cultures, to a greater or lesser extent. When a full-strength MURASHIGE and SKOOG (1962) medium supplemented with 3 % sucrose, 0.1 g l^{-1} *myo*-inositol and lacking hormones was used, solasodine content was far greater than that found in the roots of an intact *S. mauritianum* plant, or any of the callus cultures, and approached that of the green berries. This culture, which grew very rapidly in a liquid medium and which could be continually subcultured, was thus a highly productive system for the synthesis of solasodine.

Of the culture types examined in this study, the hairy root system definitely seems to provide the most favourable system for the *in vitro* synthesis of solasodine. Future research to determine the ultimate viability of this system would involve further manipulation of the cultures and the bulking-up of the hairy roots to test their growth in a bioreactor system.

Overall, this study has indicated that suspension cultures of *S. mauritianum* are not suitable for the *in vitro* synthesis of solasodine, whereas hairy root cultures are. Thus, although the approach was, of necessity, empirical, such studies can ultimately give a good indication of the options that would be worth exploring further, for possible future exploitation.

REFERENCES

AGARWAL,N. and BANSAL,Y.K. 1989. Initiation and maintenance of callus from Tendu (*Diospyros melanoxylon* Roxb.) through tissue culture. *Mendel* 6: 55-58.

AHMAD,V., ALI,S.F. and AHMAD,R. 1980. Isolation of solamargine from *Solanum albicaule*. *Planta Medica* 39: 186-191.

ALLAN, E. 1991. Plant cell culture. *In*: A.Stafford and G.Warren (eds). *Plant Cell and Tissue Culture*. Milton Keynes: Open University Press. 1-24.

ALVAREZ,M.A., NIGRA,H.M. and GIULIETTI,A.M. 1993. Solasodine production by *Solanum eleagnifolium* Cav. *in vitro* cultures: Influence of plant growth regulators, age and inoculum size. *Natural Product Letters* 2: 9-19.

ANDERSON,L.A., PHILLIPSON,J.D. and ROBERTS,M.F. 1986. Aspects of alkaloid production by plant cell cultures. *In:* P.Morris, A.H.Scragg, A.Stafford and M.W.Fowler (eds). *Secondary Metabolism in Plant Cell Cultures*. Cambridge: Cambridge University Press. 1-14.

AULD,B.A. and MEDD,R.W. 1987. *Weeds: An Illustrated Botanical Guide to the Weeds of Australia*. Melbourne: Inkata Press.

BAKER,D.C., KEELER,R.F. and GAFFIELD,W. 1989. Pathology in hamsters administered *Solanum* plant species that contain steroidal alkaloids. *Toxicon* 12: 1331-1337.

BANERJEE.S., SCHWEMMIN,A.M. and SCHWEMMIN,D.J. 1985. *In vitro* propagation of *Solanum sarrachoides* Sendt. *Journal of Plant Physiology* 119: 461-466.

BAUMERT,A., GRÖGER,D., KUZOVKINA,I.N. and REISCH,J. 1992. Secondary metabolites produced by callus cultures of various *Ruta* species. *Plant Cell, Tissue and Organ Culture* 28: 159-162.

BECKER,H. 1987. Regulation of secondary metabolism in plant cell cultures. *In:* C.E.Green, D.A.Somers, W.P.Hackett and D.D.Biesboer (eds). *Plant Tissue and Cell Culture*. New York: Alan R. Liss. 199-212.

BELL,E.A. 1981. The physiological role(s) of secondary (natural) products. *In:* E.E.Conn (ed.). *The Biochemistry of Plants - A Comprehensive Treatise. Volume 7. Secondary Plant Products*. New York: Academic Press. 1-20.

BERLIN,J., FECKER,L., RÜGENHAGEN,C., SATOR,C., STRACK,D., WITTE,L. and WRAY,V. 1991. Isoflavone glycoside formation in transformed and non-transformed suspension and hairy root cultures of *Lupinus polyphyllus* and *Lupinus hartwegii*. *Zeitschrift für Naturforschung* 46: 725-734.

BEZBURUAH,B. 1981. A simple method for purification of glycoalkaloids in the quantitative estimation of *Solanum khasianum*. *Planta Medica* 43: 77-81.

BHARATI,P. and CHATTERJEE, S.K. 1986. Agroeconomics of *Solanum laciniatum* Ait. - a new medicinal plant in Darjeeling Hills. *Acta Horticulturae* 188: 253-260.

BHARATI,P., PANDA,P and CHATTERJEE, S. 1986. Studies on improvement of some steroid yielding plants growing in Darjeeling Hills (India). *Acta Horticulturae* 188: 125-134.

BHATT,P.N., BHATT,D.P. and SUSSEX,I. 1983. Studies on some factors affecting solasodine contents in tissue cultures of *Solanum nigrum*. *Physiologia Plantarum* 57: 159-162.

BIRNER,J. 1969. Determination of total steroid bases in *Solanum* species. *Journal of Pharmaceutical Sciences* 58: 258-259.

BLOM,T.J.M., KREIS,W., VAN IREN,F. and LIBBENGA,K.R. 1992. A non-invasive method for the routine estimation of fresh weight of cells grown in batch suspension cultures. *Plant Cell Reports* 11: 146-149.

BRADLEY,V., COLLINS,D.J., CRABBE,P.G., EASTWOOD,F.W., IRVINE,M.C., SWAN,J.M. and SYMON,D.E. 1978. A survey of Australian *Solanum* plants for potentially useful sources of solasodine. *Australian Journal of Botany* 26: 723-754.

BRADLEY,V., COLLINS,D.J., EASTWOOD,F.W., IRVINE,M.C., SWAN,J.M. and SYMON,D.E. 1979. Distribution of steroidal alkaloids in Australian species of *Solanum*. *In:* J.G.Hawkes, R.N.Lester and A.D.Skelding (eds). *The Biology and Taxonomy of the Solanaceae*. London: Academic Press. 203-209.

BRAMBLE,J.L., GRAVES,D.J. and BRODELIUS,P. 1990. Plant cell culture using a novel bioreactor: The magnetically stabilized fluidized bed. *Biotechnological Progress* 6: 452-457.

BRIGGS,L.H., HARVEY,W.E., LOCKER,R.H., MCGILLIVRAY,W.A. and SEELYE,R.N. 1950. *Solanum* alkaloids. Part V. Solasodine. *Journal of the*

Chemical Society 3013-3020.

BRODELIUS,P. 1985. The potential role of immobilization in plant cell biotechnology. *Trends in Biotechnology* 3: 280-285.

BRODELIUS,P. and MOSBACH,K. 1982. Immobilized plant cells. *Applied Biochemistry and Biotechnology* 7: 31-34.

BUCHANAN,R.A., OTEY,F.H. and BAGBY,M.O. 1980. Botanochemicals. *Recent Advances in Phytochemistry* 14: 1-22.

BUITELAAR,R.M. and TRAMPER,J. 1992. Strategies to improve the production of secondary metabolites with plant cell cultures: a literature review. *Journal of Biotechnology* 23: 111-141.

BU'LOCK, J.D. 1980. Mycotoxins as secondary metabolites. *In*: P.S.Steyn (ed.). *The Biosynthesis of Mycotoxins*. New York: Academic Press. 1-17.

BUSHWAY,R.J., BUREAU,J.L. and KING,J. 1986. Modification of the rapid high performance liquid chromatographic method for the determination of potato glycoalkaloids. *Journal of Agricultural and Food Chemistry* 34: 277-279.

BUSHWAY,R.J., SAVAGE,S.A. and FERGUSON,B.S. 1987. Inhibition of acetylcholinesterase by solanaceous glycoalkaloids and alkaloids. *American Potato Journal* 64: 409-420.

CAMPBELL,P.L. 1990. Reproductive potential of *Solanum mauritianum* Scop. - Implications for control. Ph.D thesis, University of Natal.

CAMPBELL,P.L. and VAN STADEN,J. 1983. Germination of seeds of *Solanum mauritianum*. *South African Journal of Botany* 2: 301-304.

CAMPBELL,P.L. and VAN STADEN,J. 1990. Utilisation of solasodine from fruits for long-term control of *Solanum mauritianum*. *South African Forestry Journal* 155: 57-60.

CARLE,R. and REINHARD,E. 1980. Simultaneous determination of solasodine, diosgenin and tigogenin. *Planta Medica* 38: 381-383.

CARMAN,A.S., KUAN,S.S., WARE,G.M., FRANCIS,O.J. and KIRSCHENHEUTER,G.P. 1986. Rapid high-performance liquid chromatographic determination of the potato glycoalkaloids α-solanine and α-chaconine. *Journal of Agricultural and Food Chemistry* 34: 279-282.

CHAM,B.E., GILLIVER,M. and WILSON,L. 1987. Antitumour effects of glycoalkaloids isolated from *Solanum sodomaeum*. *Planta Medica* 53: 34-36.

CHAM,B.E. and WILSON,L. 1987. HPLC of glycoalkaloids from *Solanum sodomaeum*. *Planta Medica* 53: 59-62.

CHANDLER,S.F. and DODDS,J.H. 1983. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of *Solanum laciniatum*. *Plant Cell Reports* 2: 205-208.

CHAUDHURI,R.K. and CHATTERJEE,S.K. 1979. Effect of altitude and gibberellin-feeding on solasodine contents in *Solanum khasianum* Clarke, fruits of Darjeeling Hills. *Indian Journal of Experimental Biology* 17: 611-612.

CHRIQUI,D., DAVID,C. and ADAMS,S. 1988. Effect of the differentiated or dedifferentiated state of tobacco pith tissue on its behaviour after inoculation with *Agrobacterium rhizogenes*. *Plant Cell Reports* 7: 111-114.

CHRISTEN,P., AOKI,T. and SHIMOMURA,K. 1992. Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A_4. *Plant Cell Reports* 11: 597-600.

COHEN,D. 1986. The influence of explant source on the establishment of plant tissue cultures. *New Zealand Journal of Technology* 2: 95-97.

COLLIN,H.A. 1987. Determinants of yield of secondary products in plant tissue cultures. *Advances in Botanical Research* 13: 145-187.

COLLINGE,M. 1986. Ways and means to plant secondary metabolites. *Trends in Biotechnology* 4: 299-301.

COLOMBO,M.L. and TOME,F. 1991. Production of sanguinarine by *Chelidonium majus* callus cultures. *Planta Medica* 57: 428-429.

CONNER,A.J. 1982. Tissue culture of *Solanum laciniatum*. *New Zealand Journal of Botany* 20: 1-6.

CONNER,A.J. 1987. Differential solasodine accumulation in photoautotrophic and heterotrophic tissue cultures of *Solanum laciniatum*. *Phytochemistry* 26: 2749-2750.

CONSTABEL,F. 1988. Principles underlying the use of plant cell fermentation for secondary metabolite production. *Biochemistry of Cell Biology* 66: 658-664.

CONSTABEL,C.P. and TOWERS,G.N.H. 1988. Thiarubrine accumulation in hairy root cultures of *Chaenactis douglasii*. *Journal of Plant Physiology* 133: 67-72.

CORCHETE,M.P., SANCHEZ,J.M., CACHO,M., MORAN,M. and FERNANDEZ-TARRAGO,J. 1990. Cardenolide content in suspension cell cultures derived from root and leaf callus of *Digitalis thapsi* L. *Journal of Plant Physiology* 137: 196-200.

CORCUERA,L.J., HINTZ,M. and PAHLICH,E. 1989. Proline metabolism in *Solanum tuberosum* cell suspension cultures under water stress. *Journal of Plant Physiology* 134: 290-293.

CORDELL,G.A. 1981. *Introduction to Alkaloids: A biogenetic approach*. New York: John Wiley and Sons.

COXON,D.T. 1984. Methodology for glycoalkaloid analysis. *American Potato Journal* 61: 169-182.

CRABBE,P.G. and FRYER,C. 1980. Rapid quantitative analysis of solasodine, solasodine glycosides and solasodiene by high-pressure liquid chromatography. *Journal of Chromatography* 187: 87-100.

CRABBE,P.G. and FRYER,C. 1982. Evaluation of chemical analysis for the determination of solasodine in *Solanum laciniatum*. *Journal of Pharmaceutical Sciences* 71: 1356-1362.

CURTIN,M.E. 1983. Harvesting profitable products from plant tissue culture. *Bio/technology* 1: 649-657.

CURTIS,W.R., HASEGAWA,P.M. and EMERY,A.H. 1991. Modeling linear and variable growth in phosphate limited suspension cultures of opium poppies. *Biotechnology and Bioengineering* 38: 371-379.

DAVID,C. and TEMPÉ,J. 1988. Genetic transformation of cauliflower (*Brassica oleracea* L. var. *Botrytis*) by *Agrobacterium rhizogenes*. *Plant Cell Reports* 7: 88-91.

DAWSON,R.F. 1991. Diosgenin production in North America. A brief history. *HortTechnology* 1: 22-27.

DE CLEENE,M. and DE LEY,J. 1981. The host range of infectious hairy root. *The Botanical Review* 47: 147-194.

DE-EKNAMKUL,W. and ELLIS,B.E. 1985. Effects of macronutrients on growth

and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. *Plant Cell Reports* 4: 46-49.

DELFEL,N.E. 1980. The effect of nutritional factors on alkaloid metabolism in *Cephalotaxus harringtonia* tissue cultures. *Planta Medica* 39: 168-179.

DENO,H., YAMAGATA,H., EMOTO,T., YOSHIOKA,T., YAMADA,Y. and FUJITA,Y. 1987. Scopolamine production by root cultures of *Duboisia myoporoides*. II. Establishment of a hairy root culture by infection with *Agrobacterium rhizogenes*. *Journal of Plant Physiology* 131: 315-323.

DEUS-NEUMANN,B. and ZENK,M.H. 1984. Instability of indole alkaloid production in *Catharanthus roseus* cell suspension cultures. *Planta Medica* 50: 427-433.

DINNELLA,C., CRUES,J.A., D'ORAZI,D., ENCUENTRA,A., GAVALDA,E.G. and SERAFINI-FRACASSINI,D. 1992. Quantification of polyamine losses during manipulation and assay procedures. *Phytochemical Analysis* 3: 110-116.

DODDS,J.H. and ROBERTS,L.W. 1985. *Experiments in Plant Tissue Culture (2nd edition)*. Cambridge: Cambridge University Press.

DOERK,K., WITTE,L. and ALFERMANN,A.W. 1991. Identification of tropane alkaloids in hairy root cultures of *Hyoscyamus albus*. *Zeitschrift für Naturforschung* 46: 519-521.

DÖPKE,W., MATOS,N. and DUDAY,S. 1987. Über den Steroidalkaloid- und Sapogeningehalt von *Solanum panduraeforme* E. Mey. *Pharmazie* 42: 621-622.

DÖPKE,W., MOLA,J.L., MORAN,L., PALOMINO,Z., BASTERECHEA,M. and COLL,F. 1984. Über den Steroid- und Steroidalkaloidgehalt der Früchte von *Solanum mammosum* Linn. *Pharmazie* 39: 784-785.

DOUGALL,D.K. 1981. Tissue culture and the study of secondary (natural) products. *In:* E.E.Conn (ed.). *The Biochemistry of Plants - A comprehensive treatise. Volume 7. Secondary Plant Products*. New York: Academic Press. 21-34.

DOUGALL,D.K. 1986. Accumulation of natural products in plant tissue cultures revisited. *New Zealand Journal of Technology* 2: 71-76.

DREWES,F.E., VAN STADEN,J. and DREWES,S.E. 1992. Benzoylation and high performance liquid chromatographic detection of solasodine.

Phytochemical Analysis 3: 85-87.

EMKE,A. and EILERT,U. 1986. Steroidal alkaloids in tissue cultures and regenerated plants of *Solanum dulcamara*. *Plant Cell Reports* 5: 31-34.

ENDO,T., GOODBODY,A. and MISAWA,M. 1987. Alkaloid production in root and shoot cultures of *Catharanthus roseus*. *Planta Medica* 53: 479-482.

ENDO,T. and YAMADA,Y. 1985. Alkaloid production in cultured roots of three species of *Duboisia*. *Phytochemistry* 24: 1233-1236.

ERICKSON,P.I., CELLO,L.M., FROELICH,L.W. and BAHR,J.T. 1990. Rhizogenic response of tomato genotypes to *Agrobacterium rhizogenes* inoculation. *Journal of Horticultural Science* 65: 333-337.

FIGUEIREDO,A.C.S. and PAIS,M.S.S. 1991. *Achillea millefolium* (yarrow) cell suspension cultures: Establishment and growth conditions. *Biotechnology Letters* 13: 63-68.

FITZPATRICK,F.A. and SIGGIA,S. 1973. High resolution liquid chromatography of derivatized non-ultraviolet absorbing hydroxy steroids. *Analytical Chemistry* 45: 2310-2314.

FLORES,H. and FILNER,P. 1985. "Hairy roots" of Solanaceae as a source of alkaloids. *Plant Physiology* 77. Suppl.4: 12.

FLORES,H.E. and GALSTON,A.W. 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. *Plant Physiology* 69: 701-706.

FORTIN,C., NESTER,E.W. and DION,P. 1992. Growth inhibition and loss of virulence in cultures of *Agrobacterium tumefaciens* treated with acetosyringone. *Journal of Bacteriology* 174: 5676-5685.

FOWLER,M.W. 1985. Plant cell culture and natural product synthesis: An academic dream or a commercial possibility? *BioEssays* 3: 172-175.

FOWLER,M.W. 1986. Physiological factors affecting product yield in plant cell cultures. *Pesticide Science* 17: 595-601.

FRIEDMAN,M. and DAO,L. 1990. Distribution of glycoalkaloids in potato plants and commercial potato products. *Journal of Agricultural and Food Chemistry* 40: 419-423.

FRIEDMAN,M. and LEVIN,C.E. 1992. Reversed-phase high-performance liquid chromatographic separation of potato glycoalkaloids and hydrolysis products

on acidic columns. *Journal of Agricultural and Food Chemistry* 40: 2157-2163.

FROHN,D. and PFÄNDER,H.J. 1984. *A Colour Atlas of Poisonous Plants*. London: Wolfe Publishing.

FUJITA,Y. and TABATA,M. 1987. Secondary metabolites from plant cells - pharmaceutical applications and progress in commercial production. *In:* C.E.Green, D.A.Somers, W.P.Hackett and D.D.Biesboer (eds). *Plant Tissue and Cell Culture*. New York: Alan R. Liss. 169-186.

FURUYA,T., KOGE,K. and ORIHARA,Y. 1990. Long term culture and caffeine production of immobilized coffee (*Coffea arabica* L.) cells in polyurethane foam. *Plant Cell Reports* 9: 125-128.

FURUYA,T., YOSHIKAWA,T., ISHII,T. and KAJII,K. 1983. Regulation of saponin production in callus cultures of *Panax ginseng*. *Planta Medica* 47: 200-204.

GAFFIELD,W., KEELER,R.F. and BAKER,D.C. 1991. Solanum glycoalkaloids: Plant toxins possessing disparate physiologically active structural entities. *In*: R.F.Keeler and A.T.Tu (eds). *Handbook of Natural Toxins. Volume 6.* New York: Marcel Dekker, Inc. 135-158.

GAMBORG,O.L., MILLER,R.A. and OJIMA,K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151-158.

GBILE,Z.O. 1979. *Solanum* in Nigeria. *In:* J.G.Hawkes, R.N.Lester and A.D.Skelding (eds). *The Biology and Taxonomy of the Solanaceae*. London: Academic Press. 113-120.

GEISSMAN,T.A. and CROUT,D.H.G. 1969. *Organic Chemistry of Secondary Plant Metabolism*. San Francisco: Freeman, Cooper and Company.

GELVIN,S.B. 1990. Crown gall disease and hairy root disease. A sledgehammer and a tackhammer. *Plant Physiology* 92: 281-285.

GLEDDIE,S., KELLER,W.A. and SETTERFIELD,G. 1985. Plant regeneration from tissue, cell and protoplast cultures of several wild *Solanum* species. *Journal of Plant Physiology* 119: 405-418.

GOODWIN,T.W. and MERCER,E.I. 1983. *Introduction to Plant Biochemistry*. Oxford: Pergamon.

GRÄNICHER,F., CHRISTEN,P. and KAPÉTANIDIS,I. 1992. High-yield production of valepotriates by hairy root cultures of *Valeriana officinalis* L. var *sambucifolia* Mikan. *Plant Cell Reports* 11: 339-342.

GRESSEL,J. 1980. Uses and drawbacks of cell cultures in pesticide research. *In*: F.Sala, B.Parisi, R. Cella and O.Ciferri. (eds). *Plant Cell Cultures: Results and Perspectives*. Amsterdam: Elsevier. 379-388.

GUPTA,U. and BASU,P.K. 1981. A new method for quantitative determination of solasonine from developing berries of *Solanum khasianum* Clarke. *Indian Journal of Experimental Biology* 19: 1205-1206.

GURNEY,K.A. EVANS,L.V. and ROBINSON,D.S. 1992. Purine alkaloid production and accumulation in cocoa callus and suspension cultures. *Journal of Experimental Botany* 43: 769-775.

HAIGH,J.R. and LINDEN,J.C. 1989. Phenolics production by encapsulated *Nicotiana tabacum* cells. *Plant Cell Reports* 8: 475-478.

HAMILL,J.D., PARR,A.J., RHODES,M.J.C., ROBINS,R.J. and WALTON,N.J. 1987. New routes to plant secondary products. *Bio/technology* 5: 800-804.

HAMILL,J.D., PARR,A.J., ROBINS,R.J. and RHODES,M.J.C. 1986. Secondary product formation by cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes*. *Plant Cell Reports* 5: 111-114.

HARA,Y., MORIMOTO,T. and FUJITA,Y. 1987. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. V. Differences in the production between callus and suspension cultures. *Plant Cell Reports* 6: 8-11.

HARRISON,D.M. 1990. Steroidal alkaloids. *Natural Product Reports* 7: 139-147.

HASHIMOTO,T., YUKIMUNE,Y. and YAMADA,Y. 1986. Tropane alkaloid production in *Hyoscyamus niger* root cultures. *Journal of Plant Physiology* 124: 61-75.

HASLAM,E. 1985. *Metabolites and Metabolism*. Oxford: Clarendon Press.

HASLAM,E. 1986. Secondary metabolism - Fact and fiction. *Natural Product Reports* 3: 217-249.

HAUTH,S. and BEIDERBECK,R. 1992. *In vitro* culture of *Agrobacterium rhizogenes*-induced hairy roots of *Salix alba* L. *Silvae Genetica* 41: 46-48.

HEBLE,M.R., NARAYANASWAMI,S. and CHADHA,M.S. 1971. Hormonal control of steroid synthesis in *Solanum xanthocarpum* tissue culture. *Phytochemistry* 10: 2393-2394.

HEFTMANN,E. 1973. Steroids. *In:* L.P.Miller (ed.). *Phytochemistry. Volume II.* New York: Van Nostrand Reinhold. 171-226.

HEINSTEIN,P.F. 1986. Plant cell suspension cultures as a source of drugs. *Pharmacy International* 7: 38-40.

HEISER,C.B. 1969. *Nightshades: The Paradoxical Plants*. San Francisco: Freeman.

HERBRETEAU-LEMONNIER,C., ROSSIGNOL,L., ROSSIGNOL,M., SIHACHAKR,D. and HAICOUR,R. 1989. Solasodine and pigment contents of plants derived from callus cultures of *Solanum laciniatum* Ait. *Phytomorphology* 39: 93-96.

HESLOP-HARRISON,J. and HESLOP-HARRISON,Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. *Stain Technology* 45: 115-120.

HINO,F., OKAZAKI,M. and MIURA,Y. 1982. Effects of kinetin on formation of scopoletin and scopolin in tobacco tissue cultures. *Agricultural and Biological Chemistry* 46: 2195-2202.

HIRATA,K., YAMANAKA,A., KURANO,N., MIYAMOTO,K. and MIURA,Y. 1987. Production of indole alkaloids in multiple shoot cultures of *Catharanthus roseus* (L). G. Don. *Agricultural and Biological Chemistry* 51: 1311-1317.

HOLDEN,P.R., HOLDEN,M.A. and YEOMAN,M.M. 1988. Variation in the secondary metabolism of cultured plant cells. *In*: R.J.Robins and M.J.C.Rhodes (eds). *Manipulating Secondary Metabolism in Culture*. Cambridge: Cambridge University Press. 15-30.

HÖSEL,W. 1981. Glycosylation and glycosidases. *In:* E.E.Conn (ed.). *The Biochemistry of Plants - A comprehensive Treatise. Volume 7. Secondary Plant Products*. New York: Academic Press. 725-791.

HOSODA,N., ITO,H. and YATAZAWA,M. 1979. Some accounts on culture conditions of callus tissues of *Solanum laciniatum* Ait. for producing solasodine. *Agricultural and Biological Chemistry* 43: 1745-1748.

HOSODA,N. and YATAZAWA,M. 1979. Sterols, steroidal sapogenin and

steroidal alkaloid in callus culture of *Solanum laciniatum* Ait. *Agricultural and Biological Chemistry* 43: 821-825.

HOSOKI,T., KIGO,T. and SHIRAISHI,K. 1991. Transformation and plant regeneration of broccoli (*Brassica oleracea* var. italica) mediated by *Agrobacterium rhizogenes*. *Journal of the Japanese Society of Horticultural Science* 60: 71-75.

HUANG,C.-N., CORNEJO,M.J., BUSH,D.S. and JONES,R.L. 1986. Estimating viability of plant protoplasts using double and single staining. *Protoplasma* 135: 80-87.

ICHI,T., KODA,T., ASAI,I., HATANAKA,A. and SEKIYA,J. 1986. Effects of gelling agents on *in vitro* culture of plant tissues. *Agricultural and Biological Chemistry* 50: 2397-2399.

IMOTO,S. and OHTA,Y. 1988. Elicitation of diacetylenic compounds in suspension cultured cells of eggplant. *Plant Physiology* 86: 176-181.

INDRAYANTO,G., CHOLIES,N. and WAHYUDI,Y. 1985. Influence of fruit size of *Solanum wrightii* on its solasodine content. *Planta Medica* 51: 470.

INDRAYANTO,G. and SUTARJADI,I. 1986. Sterols in callus cultures of *Solanum mammosum*. *Planta Medica* 52: 413.

INOMATA,S., YOKOYAMA,M., SETO,S. and YANAGI,M. 1991. High-level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseus* plant cells. *Applied Microbiology and Biotechnology* 36: 315-319.

ISHIDA,B.K. 1988. Improved diosgenin production in *Dioscorea deltoidea* cell cultures by immobilization in polyurethane foam. *Plant Cell Reports* 7: 270-273.

ISHIMARU,K. and SHIMOMURA,K. 1991. Tannin production in hairy root culture of *Geranium thunbergii*. *Phytochemistry* 30: 825-828.

ISOGAI,A., FUKUCHI,N., HAYASHI,M., KAMADA,H., HARADA,H. and SUZUKI,A. 1990. Mikimopine, an opine in hairy roots of tobacco induced by *Agrobacterium rhizogenes*. *Phytochemistry* 29: 3131-3134.

JAGGI,R.K., BHATNAGAR,J.K., QADRY,J.S. and KAPOOR,V.K. 1987. Static callus culture of *Solanum platanifolium*. *Indian Drugs* 25: 98-101.

JAIN,S.C. and SAHOO,S. 1981a. Growth and production of steroidal sapogenins

and glycoalkaloids in *Solanum jasminoides* Paxt., suspension cultures. *Agricultural and Biological Chemistry* 45: 2909-2910.

JAIN,S.C. and SAHOO,S. 1981b. Isolation and characterization of steroidal sapogenins and glycoalkaloids from tissue cultures of *Solanum verbascifolium* Linn. *Chemical and Pharmaceutical Bulletin* 29: 1765-1767.

JAIN,S.C. and SAHOO,S. 1986. Isolation of steroids and glycoalkaloids from *Solanum glaucophyllum* Desf. *Pharmazie* 41: 820-821.

JHA,S., SAHU,N.P., SEN,J., JHA,T.B. and MAHATO,S.B. 1991. Production of emetine and cephaeline from cell suspension and excised root cultures of *Cephaelis ipecacuanha*. *Phytochemistry* 30: 3999-4003.

JOHNSON,E.L. and STEVENSON,R. 1978. *Basic Liquid Chromatography*. Palo Alto, California: Varian Associates.

JONKER,H.H., KOOPS,A.J. and HOOGENDOORN,J.C. 1992. A rapid method for the quantification of steroidal glycoalkaloids by reversed phase HPLC. *Potato Research* 35: 451-455.

JUNG,K.H., KWAK,S.S., KIM,S.W., LEE,H., CHOI,C.Y. and LIU,J.R. 1992. Improvement of the catharanthine productivity in hairy root cultures of *Catharanthus roseus* by using monosaccharides as a carbon source. *Biotechnology Letters* 14: 695-700.

KADKADE,P.G., RECINÓS,J.A. and MADRID,T.R. 1979. Studies on the distribution of glycoalkaloids in *Solanum acculeatissimum*. *Planta Medica* 37: 70-72.

KAMADA,H., OKAMURA,N., SATAKE,M., HARADA,H. and SHIMOMURA, K. 1986. Alkaloid production by hairy root cultures in *Atropa belladonna*. *Plant Cell Reports* 5: 239-242.

KAMAL,R. and KHANNA,P. 1979. Histamine from *Helianthus annuus* L. seedlings and callus cultures. *Planta Medica* 37: 181-182.

KAMEYA,T., MIYAZAWA,N. and TOKI,S. 1990. Production of somatic hybrids between *Solanum melongena* L. and *S. integrifolium* Poir. *Japanese Journal of Breeding* 40: 429-434.

KANG,K.S., VEEDER,G.T., MIRRASOUL,P.J., KANEKO,T. and COTTRELL,I.W. 1982. Agar-like polysaccharide produced by a *Pseudomonas* species: Production and its basic properties. *Agricultural and Environmental Microbiology* 43: 1086-1091.

KARTNIG,T., KUMMER-FUSTINIONI,G. and HEYDEL,B. 1983. The effect of aging on the formation of secondary products by tissue culture of *Digitalis purpurea*. *Planta Medica* 47: 247-248.

KATAVIĆ,V., JELASKA,S., BAKRAN-PETRICIOLI,T. and DAVID,C. 1991. Host-tissue differences in transformation of pumpkin (*Cucurbita pepo* L.) by *Agrobacterium rhizogenes*. *Plant Cell, Tissue and Organ Culture* 24: 35-42.

KAWAGUCHI,K., HIROTANI,M., YOSHIKAWA,T. and FURUYA,T. 1990. Biotransformation of digitoxigenin by ginseng hairy root cultures. *Phytochemistry* 29: 837-843.

KEELER,R.F., BAKER,D.C, and GAFFIELD,W. 1990. Spirosolane-containing *Solanum* species and induction of congenital craniofacial malformations. *Toxicon* 28: 873-884.

KHANNA,K.R. and SHUKLA,S. 1991. Genetics of secondary plant products and breeding for their improved content and modified quality. *In*: K.R.Khanna (ed.). *Biochemical aspects of crop improvement*. Boca Raton: CRC Press. 283-323.

KHANNA,P. and STABA,E.J. 1968. Antimicrobials from plant tissue cultures. *Lloydia* 31: 180-189.

KHANNA,P., UDDIN,A., SHARMA,G.L., MANOT,S.K. and RATHORE,A.K. 1976. Isolation and characterization of sapogenin and solasodine from *in vitro* tissue cultures of some solanaceous plants. *Indian Journal of Experimental Biology* 14: 694-696.

KHOURI,H.E., IBRAHIM,R.K. and RIDEAU,M. 1986. Effects of nutritional and hormonal factors on growth and production of anthraquinone glucosides in cell suspension cultures of *Cinchona succirubra*. *Plant Cell Reports* 5: 423-426.

KING,P.J. and STREET,H.E. 1977. Growth patterns in cell cultures. *In*: H.E.Street (ed.). *Plant Tissue and Cell Culture*. Oxford: Blackwell Scientific Publications. 269-337.

KITAMURA,Y., MIURA,H. and SUGII,M. 1985. Alkaloid composition and atropine esterase activity in callus and differentiated tissues of *Duboisia myoporoides* R.Br. *Chemical and Pharmaceutical Bulletin* 33: 5445-5448.

KITAMURA,Y., SUGIMOTO,Y., SAMEJIMA,T., HAYASHIDA,K. and MIURA,H. 1991. Growth and alkaloid production in *Duboisia myoporoides* and *D. leichhardtii* root cultures. *Chemical and Pharmaceutical Bulletin* 35:

1263-1266.

KITAMURA,Y., TAURA,A., KAJIYA,Y. and MIURA,H. 1992. Conversion of phenylalanine and tropic acid into tropane alkaloids by *Duboisia leichhardtii* root cultures. *Journal of Plant Physiology* 140: 141-146.

KLYNE,W. 1960. *The Chemistry of the Steroids*. London: Methuen & Co. Ltd.

KNOPP,E., STRAUSS,A. and WEHRLI,W. 1988. Root induction on several Solanaceae species by *Agrobacterium rhizogenes* and the determination of root tropane alkaloid content. *Plant Cell Reports* 7: 590-593.

KOGA,D., SUESHIGE,N., TAHARA,M., KATO,M. and IDE,A. 1990. Callus induction from yam, *Dioscorea opposita* Thunb., and the subculture. *Agricultural and Biological Chemistry* 54: 245-246.

KOKATE,C.K. and RADWAN,S.S. 1979. Enrichment of *Solanum tuberosum* callus generating rootlets with steroidal glycoalkaloids. *Zeitschrift für Naturforschung* 34: 634-636.

KOUL,S., AHUJA,A. and GREWAL,S. 1983. Growth and alkaloid production in suspension cultures of *Hyoscyamus muticus* as influenced by various cultural parameters. *Planta Medica* 47: 11-16.

KRUEGER,R.J., CAREW,D.P., LUI,J.H.C. and STABA,E.J. 1982. Initiation, maintenance and alkaloid content of *Catharanthus roseus* leaf organ cultures. *Planta Medica* 45: 56-57.

KUC,J. 1984. Steroid glycoalkaloids and related compounds as potato quality factors. *American Potato Journal* 61: 123-140.

KURZ,W.G.W. 1986. Biological and environmental factors of product synthesis, accumulation, and biotransformation by plant cell cultures. *New Zealand Journal of Technology* 2: 77-81.

KURZ,W.G.W. and CONSTABEL,F. 1985. Aspects affecting biosynthesis and biotransformation of secondary metabolites in plant cell cultures. *CRC Critical Reviews in Biotechnology* 2: 105-118.

KUSANO,G., TAKAHASHI,A., SUGIYAMA,K. and NOZOE,S. 1987. Antifungal properties of *Solanum* alkaloids. *Chemical and Pharmaceutical Bulletin* 35: 4862-4867.

KYO,M., MIYAUCHI,Y., FUJIMOTO,T. and MAYAMA,S. 1990. Production of nematicidal compounds by hairy root cultures of *Tagetes patula* L. *Plant Cell*

Reports 9: 393-397.

LAHA,M.K. and BASU,P.K. 1983. Biological hydrolysis of glycoalkaloids from *Solanum khasianum* by a local strain of *Aspergillus niger*. *International Journal of Crude Drug Research* 4: 153-155.

LANCASTER,J.E. and MANN,J.D. 1975. Changes in solasodine content during the development of *Solanum laciniatum* Ait. *New Zealand Journal of Agricultural Research* 18: 139-144.

LANCASTER,J.E., MANN,J.D. and BLYTH,K.E. 1977. Effect of age on amount and type of glycoalkaloids in leaves and roots of *Solanum laciniatum* Ait. *New Zealand Journal of Agricultural Research* 20: 395-399.

LAWSON,D.R., ERB,W.A. and MILLER,A.R. 1992. Analysis of *Solanum* alkaloids using internal standardization and capillary gas chromatography. *Journal of Agricultural and Food Chemistry* 40: 2186-2191.

LIN,C.-N. and GAN,K.-H. 1989. Antihepatotoxic principles of *Solanum capsicastrum*. *Planta Medica* 55: 48-50.

LUCKNER,M. 1984. *Secondary Metabolism in Microorganisms, Plants and Animals. (2nd edition).* Berlin: Springer Verlag.

MACEK,T.E. 1989. XXV. *Solanum aviculare* Forst., *Solanum laciniatum* Ait. (Poroporo): *In vitro* culture and the production of solasodine. *In:* Y.P.S.Bajaj (ed.). *Biotechnology in Agriculture and Forestry, Volume 7. Medicinal and Aromatic Plants II.* Berlin: Springer Verlag. 443-467.

MACRAE,S. and VAN STADEN,J. 1990. *In vitro* culture of *Eucalyptus grandis*: Effect of gelling agents on propagation. *Journal of Plant Physiology* 137: 249-251.

MANDAL,S. and GADGIL,V.N. 1979. Differentiation of shoot buds and roots in fruit callus culture of *Solanum nigrum*. *Indian Journal of Experimental Biology* 17: 876-878.

MANN,J.D. 1978. Production of solasodine for the pharmaceutical industry. *Advances in Agronomy* 30: 207-245.

MANN, J. 1987. *Secondary Metabolism (2nd edition).* Oxford: Clarendon Press.

MANO,Y., NABESHIMA,S., MATSUI,C. and OHKAWA,H. 1986. Production of tropane alkaloids by hairy root cultures of *Scopolia japonica*. *Agricultural and Biological Chemistry* 50: 2715-2722.

MANO,Y., OHKAWA,H. and YAMADA,Y. 1989. Production of tropane alkaloids by hairy root cultures of *Duboisia leichhardtii* transformed by *Agrobacterium rhizogenes*. *Plant Science* 59: 191-201.

MATHÉ,I.(Jr), MATHÉ,I., BOTZ,L. and KOCH,L. 1986. Possibilities of solanum alkaloid production in European temperate zone. *Acta Horticulturae* 188: 193-201.

MATHEWS,H., BHARATHAN,N., LITZ,R.E., NARAYANAN,K.R., RAO,P.S. and BHATIA,C.R. 1990. The promotion of *Agrobacterium* mediated transformation in *Atropa belladonna* L. by acetosyringone. *Journal of Plant Physiology* 136: 404-409.

MATSUMOTO,T. and TANAKA,N. 1991. Production of phytoecdysteroids by hairy root cultures of *Ajuga reptans* var. *atropurpurea*. *Agricultural and Biological Chemistry* 55: 1019-1025.

MATTOS,N.DE O. and CORDEIRO,A.R. 1989. "In vitro" plant regeneration from leaf explants of *Solanum viarum* Dun. and *S. sisymbrifolium* Lam. *Revista Brasiliera de Biologia* 49: 27-32.

MAVITUNA,F. and PARK.J.M. 1985. Growth of immobilised plant cells in reticulate polyurethane foam matrices. *Biotechnology Letters* 7: 637-640.

MIKI,B.L.A. and IYER,V.N. 1990. Fundamentals of gene transfer in plants. *In*: D.T.Dennis and D.H.Turpin (eds). *Plant Physiology, Biochemistry and Molecular Biology*. London: Longman Scientific and Technical. 473-489.

MILLER,L.M.F. and DAVIES,M.E. 1978. The characteristics of solasodine accumulation in *Solanum khasianum* C. B. Clarke var. *chatterjeeanum* Sengupta and *S. laciniatum* Ait. grown under field conditions in Birmingham. *In:* J.G.Hawkes, R.N.Lester and A.D.Skelding (eds). *The Biology and Taxonomy of the Solanaceae*. London: Academic Press. 231-235.

MORRIS,P. 1986a. Regulation of product synthesis in cell cultures of *Catharanthus roseus*. I. Effect of culture temperature. *Plant Cell Reports* 5: 427-429.

MORRIS,P. 1986b. Regulation of product synthesis in cell cultures of *Catharanthus roseus*. III. Alkaloid metabolism in cultured leaf tissue and primary callus. *Planta Medica* 52: 127-132.

MORRIS,V.J., BROWNSEY,G.J., GUNNING,A.P. and HARRIS,J.E. 1990. Gelation of the extracellular polysaccharide produced by *Agrobacterium rhizogenes*. *Carbohydrate Polymers* 13: 221-225.

MORRIS,S.C. and LEE,T.H. 1984. The toxicity and teratogenicity of Solanaceae glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): A review. *Food Technology in Australia* 36: 118-124.

MOUMOU,Y., TROTIN,F., DUBOIS,J., VASSEUR,J. and EL-BOUSTANI,E. 1992. Influence of culture conditions on polyphenol production by *Fagopyrum esculentum* tissue cultures. *Journal of Natural Products* 55: 33-38.

MOUMOU,Y., VASSEUR,J., TROTIN,F. and DUBOIS,J. 1992. Catechin production by callus cultures of *Fagopyrum esculentum*. *Phytochemistry* 31: 1239-1241.

MUGNIER,J. 1988. Establishment of new axenic hairy root lines by inoculation with *Agrobacterium rhizogenes*. *Plant Cell Reports* 7: 9-12.

MULDER-KRIEGER,T., VERPOORTE,R., VAN DER KREEK,M. and BAERHEIM-SVENDSEN,A. 1984. Identification of alkaloids and anthraquinones in *Cinchona pubescens* callus cultures; the effect of plant growth regulators and light on the alkaloid content. *Planta Medica* 50: 17-20.

MUNDAY,J. 1988. *Poisonous Plants in Southern African Gardens and Parks*. Craighall: Delta Books.

MURAKAMI,K., EZIMA,H., TAKAISHI,Y., TAKEDA,Y., FUJITA,T., SATO,A., NAGAYAMA,Y. and NOHARA,T. 1985. Studies on the constituents of *Solanum* plants. V. The constituents of *S. lyratum* Thunb. II. *Chemical and Pharmaceutical Bulletin* 33: 67-73.

MURASHIGE,T. and SKOOG,F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

NACHTMANN,F., SPITZY,H. and FREI,R.W. 1976a. Rapid and sensitive high-resolution procedure for digitalis glycoside analysis by derivatization liquid chromatography. *Journal of Chromatography* 122: 293-303.

NACHTMANN,F., SPITZY,H. and FREI,R.W. 1976b. Ultraviolet derivatization of *Digitalis* glycosides as 4-nitro benzoates for liquid chromatographic trace analysis. *Analytical Chemistry* 48: 1576-1579.

NAIR,A.J., SUDHAKARAN,P.R., RAO,J.M. and RAMAKRISHNA,S.V. 1992. Berberine synthesis by callus cultures and cell suspension cultures of *Coscinium fenestratum*. *Plant Cell, Tissue and Organ Culture* 29: 7-10.

NAKAGAWA,K., FUKUI,H. and TABATA,M. 1986. Hormonal regulation of berberine production in cell suspension cultures of *Thalictrum minus*. *Plant Cell Reports* 5: 69-71.

NGUYEN,C., BOURGAUD,F., FORLOT,P. and GUCKERT,A. 1992. Establishment of hairy root cultures of *Psoralea* species. *Plant Cell Reports* 11: 424-427.

NIGRA,H.M., ALVAREZ,M.A. and GIULIETTI,A.M. 1989. The influence of auxins, light and cell differentiation on solasodine production by *Solanum eleagnifolium* Cav. calli. *Plant Cell Reports* 8: 230-233.

NIGRA,H.M., ALVAREZ,M.A. and GIULIETTI,A.M. 1990. Effect of carbon and nitrogen sources on growth and solasodine production in batch suspension cultures of *Solanum eleagnifolium* Cav. *Plant Cell, Tissue and Organ Culture* 21: 55-60.

NIGRA,H.M., CASO,O.H. and GIULIETTI,A.M. 1987. Production of solasodine by calli from different parts of *Solanum eleagnifolium* Cav. plants. *Plant Cell Reports* 6: 135-137.

NODA,T., TANAKA,N., MANO,Y., NABESHIMA,S., OHKAWA,H. and MATSUI,C. 1987. Regeneration of horseradish hairy roots incited by *Agrobacterium rhizogenes* infection. *Plant Cell Reports* 6: 283-286.

ODDO, G. and COLOMBANO, A. 1905. Über die Produkte, die man aus *Solanum sodomaeum* Linn. extrahiert. *Chemische Berichte* 38: 2755-2758.

ODNEVALL,A. and BJÖRK,L. 1989. Differential tissue cultures of *Panax ginseng* and their response to various carbon sources. *Biochemie und Physiologie der Pflanzen* 185: 403-413.

OHKAWA,H., KAMADA,H., SUDO,H. and HARADA,H. 1989. Effects of gibberellic acid on hairy root growth in *Datura innoxia*. *Journal of Plant Physiology* 134: 633-636.

OJIMA,K. and OHIRA,K. 1978. Nutritional requirements of callus and cell suspension cultures. *In*: T.A Thorpe (ed.). *Frontiers of Plant Tissue Culture*. Calgary: The International Association for Plant Tissue Culture. 265-275.

OKAZAKI,M., HINO,F., KOMINAMI,K. and MIURA,Y. 1982. Effects of plant hormones on formation of scopoletin and scopolin in tobacco tissue cultures. *Agricultural and Biological Chemistry* 46: 609-614.

OKAZAKI,M., HINO,F., NAGASAWA,K. and MIURA,Y. 1982. Effects of

nutritional factors on formation of scopoletin and scopolin in tobacco tissue cultures. *Agricultural and Biological Chemistry* 46: 601-607.

OKSMAN-CALDENTEY,K.-M., KIVELÄ,O. and HILTUNEN,R. 1991. Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. *Plant Science* 78: 129-136.

OKSMAN-CALDENTEY,K.-M., STRAUSS,A. and HILTUNEN,R. 1986. Optimization of some parameters in cultivating scopolamine-producing cells of *Hyoscyamus muticus*. *Planta Medica* 52: 513.

ONDŘEJ,M. and BÍSKOVÁ,R. 1986. Differentiation of *Petunia hybrida* tissues transformed by *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. *Biologia Plantarum* 28: 152-155.

ONDŘEJ,M. and PROTIVA,J. 1987. *In vitro* culture of crown gall callus and hairy root tumours of *Atropa belladonna*: Differentiation and alkaloid production. *Biologia Plantarum* 29: 241-246.

OOMS,G., BOSSEN,M.E., BURRELL,M.M. and KARP,A. 1986. Genetic manipulation in potato with *Agrobacterium rhizogenes*. *Potato Research* 29: 367-379.

OSMAN,S.F. 1980. Glycoalkaloids of the Solanaceae. *Recent Advances in Phytochemistry* 14: 75-96.

OSMAN,S.F., SINDEN,S.L., IRWIN,P., DEAHL,K. and TINGEY,W.M. 1991. Solanocardinol: A steroidal alkaloid from *Solanum neocardenasii*. *Phytochemistry* 30: 3161-3163.

OTTAVIANI,M.P. SCHEL,J.H.N. and HÄNISCH TEN CATE,C.H. 1990. Variation in structure and plant regeneration of *Agrobacterium rhizogenes* transformed and control roots of the potato cv. Bintje. *Plant Cell, Tissue and Organ Culture* 20: 25-34.

PANDEYA,S.C., SARATBABU,G.V. and BHATT,A.B. 1981a. Dynamics of solasodine accumulation in developing berries of *Solanum* sysimbrifolium. *Planta Medica* 42: 409-411.

PANDEYA,S.C., SARATBABU,G.V. and BHATT,A.B. 1981b. A quick method for estimation of solasodine in leaves and berries of *Solanum sysimbrifolium* Lamk. *Indian Journal of Experimental Biology* 19: 1207-1208.

PARK,C.-H. and MARTINEZ,B.C. 1992. Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by dimethylsulfoxide (DMSO) while

preserving cell viability and growth. *Biotechnology and Bioengineering* 40: 459-464.

PARR,A.J. and HAMILL,J.D. 1987. Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants. *Phytochemistry* 26: 3241-3245.

PARR,A.J., ROBINS,R.J. and RHODES,M.J.C. 1984. Permeabilization of *Cinchona ledgeriana* cells by dimethylsulphoxide. Effects on alkaloid release and long-term membrane integrity. *Plant Cell Reports* 3: 262-265.

PATIL,S. and LALORAYA,M.M. 1984. Steroidal contents of berries and roots of *Solanum viarum* Dunal. *Indian Journal of Chemistry* 23: 685-686.

PAUTHE-DAYDE,D., ROCHD,M. and HENRY,M. 1990. Triterpenoid saponin production in callus and multiple shoot cultures of *Gypsophila* spp. *Phytochemistry* 29: 483-487.

PAYNE,J., HAMILL,J.D., ROBINS,R.J. and RHODES,M.J.C. 1987. Production of hyoscyamine by "hairy root" cultures of *Datura stramonium*. *Planta Medica* 53: 474-478.

PETIT,A., DAVID,C., DAHL,G.A., ELLIS,J.C., GUYON,P., CASSE-DELBART,F. and TEMPÉ,J. 1983. Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Molecular and General Genetics* 190: 204-215.

PETTERSON,D.S., HARRIS,D.J. and ALLEN,O.G. 1991. Alkaloids. *In*: J.P.F.D'Mello, C.M.Duffus and J.H.Duffus (eds). *Toxic Substances in Crop Plants*. Cambridge: The Royal Society of Chemistry. 148-179.

PILACINSKI,W.P. and SCHMIDT,E.L. 1981. Plasmid transfer within and between serologically distinct strains of *Rhizobium japonicum*, using antibiotic resistance mutants and auxotrophs. *Journal of Bacteriology* 145: 1025-1030.

PRAS,N. 1992. Bioconversion of naturally occurring precursors and related synthetic compounds using plant cell cultures. *Journal of Biotechnology* 26: 29-62.

PRELOG,V. and JEGER,O. 1953. The chemistry of *Solanum* and *Veratrum* alkaloids. *In:* R.H.F.Manske and H.L.Holmes (eds). *The Alkaloids. Chemistry and Physiology*. New York: Academic Press. 247-310.

PURI,R.K. and SHUKLA,D.C. 1979. Effects of auxins on growth and solasodine

content of *Solanum platanifolium* during ontogenesis. *Planta Medica* 36: 142-149.

QUYEN,L.T., KHOI,N.H., SUONG,N.N., SCHREIBER,K. and RIPPERGER,H. 1987. Steroid alkaloids and yamogenin from *Solanum spirale*. *Planta Medica* 53: 292-293.

REICHLING,J. and THRON,U. 1990. Accumulation of rare phenylpropanoids in *Agrobacterium rhizogenes* transformed root cultures of *Coreopsis tinctoria*. *Planta Medica* 56: 488-490.

REYNOLDS,T.L. 1987. Cytodifferentiation in cell suspension cultures of *Solanum carolinense*: Effects of 2,4-D and kinetin. *Journal of Plant Physiology* 130: 373-383.

RHODES,M.J.C., ROBINS,R.J., HAMILL,J. and PARR,A.J. 1986. Potential for the production of biochemicals by plant cell cultures. *New Zealand Journal of Technology* 2: 59-70.

RIDEAU,M. 1987. Optimisation de la production de métabolites par des cellules végétales *in vitro*. *Annales Pharmaceutiques Françaises* 45: 133-144.

RIDOUT,C.L., PRICE,K.R., COXON,D.T. and FENWICK,G.R. 1989. Glycoalkaloids from *Solanum nigrum* L., α-solamargine and α-solasonine. *Pharmazie* 44: 732-733.

RIPPERGER,H. 1990. 25-Isoetioline from two *Solanum* species. *Phytochemistry* 29: 3375-3376.

RIPPERGER,H. and PORZEL,A. 1991. (23*S*)-23-Hydroxysolasodine from two *Solanum* species. *Phytochemistry* 30: 1299-1301.

RIPPERGER,H. and PORZEL,A. 1992. N-Hydroxysolasodine from *Solanum robustum*. *Phytochemistry* 31: 1837-1839.

ROBERTS, M.F. 1988. Medicinal products through plant biotechnology. *In:* R.J.Robins and M.J.C.Rhodes (eds). *Manipulating Secondary Metabolism in Culture*. Cambridge: Cambridge University Press. 201-216.

ROBERTSON,G.H., DOYLE,L.R., SHENG,P., PAVLATH,A.E. and GOODMAN,N. 1989. Diosgenin formation by freely suspended and entrapped plant cell cultures of *Dioscorea deltoidea*. *Biotechnology and Bioengineering* 34: 1114-1125.

ROBINS,R.J., PARR,A.J., PAYNE,J., WALTON,N.J. and RHODES,M.J.C.

1990. Factors regulating tropane-alkaloid production in a transformed root culture of a *Datura candida* x *D. aurea* hybrid. *Planta* 181: 414-422.

ROBINS,R.J., PARR,A.J. and RHODES,M.J.C. 1988. The use of continuous product removal to influence the distribution and net production of alkaloids by cell cultures. *Biochemical Society Transactions* 16: 67-71.

RODDICK,J.G. 1989. The acetylcholinesterase inhibitory activity of steroidal glycoalkaloids and their aglycones. *Phytochemistry* 28: 2631-2634.

RODDICK,J.G. and MELCHERS,G. 1985. Steroidal glycoalkaloid content of potato, tomato and their somatic hybrids. *Theoretical and Applied Genetics* 70: 655-660.

RODDICK,J.G., RIJNENBERG,A.L. and WEISSENBERG,M. 1990. Membrane-disrupting properties of the steroidal glycoalkaloids solasonine and solamargine. *Phytochemistry* 29: 1513-1518.

RODDICK,J.G., RIJNENBERG,A.L. and WEISSENBERG,M. 1992. Alterations to the permeability of liposome membranes by the solasodine-based glycoalkaloids solasonine and solamargine. *Phytochemistry* 31: 1951-1954.

RODRÍGUEZ-MENDIOLA,M.A., STAFFORD,A., CRESSWELL,R. and ARIAS-CASTRO,C. 1991. Bioreactors for growth of plant roots. *Enzyme and Microbial Technology* 13: 697-701.

ROJA,G., BENJAMIN,B.D., HEBLE,M.R., PATANKAR,A.V. and SIPAHIMALANI,A.T. 1990. The effect of plant growth regulators and nutrient conditions on growth and alkaloid production in multiple shoot cultures of *Rauwolfia serpentina*. *Phytotherapy Research* 4: 49-52.

ROSS,J.H. 1972. *The Flora of Natal*. Pretoria: Department of Agricultural Technical Services.

RYU,D.D.Y., LEE,S.O. and ROMANI,R.J. 1990. Determination of growth rate for plant cell cultures: Comparative studies. *Biotechnology and Bioengineering* 35: 305-311.

SAITOU,T., KAMADA,H. and HARADA,H. 1991. Isoperoxidase in hairy roots and regenerated plants of horseradish (*Armoracia lapathifolia*). *Plant Science* 75: 195-201.

SAMANTA,S.K. and SEN,S. 1983. X-ray and EMS induced variability in solasodine content of *Solanum khasianum* Clarke. *Zeitschrift für Pflanzenzüchtung* 91: 78-81.

SASSE,F., WITTE,L. and BERLIN,J. 1987. Biotransformation of tryptamine to seratonin by cell suspension cultures of *Peganum harmala*. *Planta Medica* 53: 354-359.

SAUERWEIN,M., ISHIMARU,K. and SHIMOMURA,K. 1991. Indole alkaloids in hairy roots of *Amsonia elliptica*. *Phytochemistry* 30: 1153-1155.

SAUERWEIN,M. and SHIMOMURA,K. 1991. Alkaloid production in hairy roots of *Hyoscyamus albus* transformed with *Agrobacterium rhizogenes*. *Phytochemistry* 30: 3277-3280.

SCHMAUDER,H.-P. and DOEBEL,P. 1990. Plant cell cultivation as a biotechnological method. *Acta Biotechnologica* 10: 501-516.

SCHREIBER,K. 1973. Synthesis and biogenesis of steroidal *Solanum* alkaloids. *Biochemical Society Transactions* 2: 1-25.

SCOTT,C.D. 1987. Immobilized cells: A review of recent literature. *Enzyme and Microbial Technology* 9: 66-73.

SCRAGG,A.H. 1986. The economics of mass cell culture. *In*: P.Morris, A.H.Scragg, A.Stafford and M.W.Fowler (eds). *Secondary Metabolism in Plant Cell Cultures*. Cambridge: Cambridge University Press. 202-207.

SCRAGG, A. 1991. The immobilization of plant cells. *In*: A.Stafford and G.Warren (eds). *Plant Cell and Tissue Culture*. Milton Keynes: Open University Press. 205-220.

SCRAGG,A.H. and ALLAN,E.J. 1986. Production of the triterpenoid quassin in callus and cell suspension cultures of *Picrasma quassioides* Bennett. *Plant Cell Reports* 5: 356-359.

SHARMA,R.P. and SALUNKHE,D.K. 1989. Solanum glycoalkaloids. *In*: P.R.Cheeke (ed.). *Toxicants of Plant Origin. Volume I. Alkaloids*. Boca Raton: CRC Press. 179-236.

SHARP,J.M, and DORAN,P.M. 1990. Characteristics of growth and tropane alkaloid synthesis in *Atropa belladonna* roots transformed by *Agrobacterium rhizogenes*. *Journal of Biotechnology* 16: 171-186.

SHEIKHOLESLAM,S.N. and WEEKS,D.P 1987. Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. *Plant Molecular Biology* 8: 291-298.

SHUM,O.L. and CHIU,K.W. 1991. Hypotensive action of *Solanum melongena* on

normotensive rats. *Phytotherapy Research* 5: 76-81.

SHUNGU,D., VALIANT,M., TUTLANE,V., WEINBERG,E., WEISSBERGER,B., KOUPAL,L., GADEBUSCH,H. and STAPLEY,E. 1983. Gelrite as an agar substitute in bacteriological media. *Applied and Environmental Microbiology* 46: 840-845.

SIAH,C.L. and DORAN,P.M. 1991. Enhanced codeine and morphine production in suspended *Papaver somniferum* cultures after removal of exogenous hormones. *Plant Cell Reports* 10: 349-353.

SINDEN,S.L., SANFORD,L.L. and OSMAN,S.F. 1980. Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. *American Potato Journal* 57: 331-343.

SMITH,R.H. 1986. Establishment of calli and suspension cultures. *Methods in Enzymology* 118: 539-549.

SMITH,J.I., QUESNEL,A.A., SMART,N.J. and MISAWA,M. 1987. The development of a single-stage growth and indole alkaloid production medium for *Catharanthus roseus* (L.) G. Don suspension cultures. *Enzyme and Microbial Technology* 9: 466-469.

SONG,Y.-N., SHIBUYA,M., EBIZUKA,Y. and SANKAWA,U. 1990. Hydroxyacetosyringone is the major virulence gene activating factor in belladonna hairy root cultures, and inositol enhances its activity. *Chemical and Pharmaceutical Bulletin (Tokyo)* 38: 2063-2065.

STAFFORD, A. 1991. Natural products and metabolites from plants and plant tissue cultures. *In*: A.Stafford and G.Warren (eds). *Plant Cell and Tissue Culture*. Milton Keynes: Open University Press. 124-162.

STILL,W.C., KAHN,M. and MITRA,A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *Journal of Organic Chemistry* 43: 2923-2924.

STREET,H.E. 1977. Cell (suspension) cultures - Techniques. *In:* H.E.Street (ed.). *Plant Tissue and Cell Culture*. Oxford: Blackwell. 59-102.

STROBEL,J., HIEKE,M., GEBAUER,E., WIND,E. and GRÖGER,D. 1990. The influence of organic and inorganic chemical factors on cell growth and anthraquinone formation in suspension cultures of *Galium vernum*. *Biochemie und Physiologie der Pflanzen* 186: 117-124.

SUZUKI,H., MATSUMOTO,T. and MIKAMI,Y. 1984. Effects of nutritional

factors on the formation of anthraquinones by *Rubia cordifolia* plant cells in suspension cultures. *Agricultural and Biological Chemistry* 48: 603-610.

SUZUKI,M., NAKAGAWA,K., FUKUI,H. and TABATA,M. 1988. Alkaloid production in cell suspension cultures of *Thalictrum flavum* and *T. dipterocarpum*. *Plant Cell Reports* 7: 26-29.

TANAKA,N., HAYAKAWA,M., MANO,Y., OHKAWA,H. and MATSUI,C. 1985. Infection of turnip and radish storage roots with *Agrobacterium rhizogenes*. *Plant Cell Reports* 4: 74-77.

TELEK,L. 1979. Preparation of solasodine from fruits of *Solanum* species. *Planta Medica* 37: 92-94.

TEPFER,D.A. and TEMPÉ,J. 1981. Production of agropine by roots transformed under the action of *Agrobacterium rhizogenes* strain A_4. *Academie des Sciences. Comptes Rendus des Seances. Ser.III* 292: 153-156.

THORNE,H.V., CLARKE,G.F. and SKUCE,R. 1985. The inactivation of herpes simplex virus by some Solanaceae glycoalkaloids. *Antiviral Research* 5: 335-343.

TOIVONEN,L., BALSEVICH,J. and KURZ,W.G.W. 1989. Indole alkaloid production by hairy root cultures of *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* 18: 79-93.

TOPPEL,G., WITTE,L., RIEBESEHL,B., VAN BORSTEL,K. and HARTMANN,T. 1987. Alkaloid patterns and biosynthetic capacity of root cultures from some pyrrolizidone alkaloid producing *Senecio* species. *Plant Cell Reports* 6: 466-469.

TRULSON,A.J., SIMPSON,R.B. and SHAHIN,E.A. 1986. Transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes*. *Theoretical and Applied Genetics* 73: 11-15.

TSOULPHA,P. and DORAN,P.M. 1991. Solasodine production from self-immobilized *Solanum aviculare* cells. *Journal of Biotechnology* 19: 99-110.

UDDIN,A. and CHATURVEDI,H.C. 1979. Solasodine in somatic tissue cultures of *Solanum khasianum*. *Planta Medica* 37: 90-92.

VÁGÚJFALVI,D., MARÓTI,M. and TÉTÉNYI,P. 1971. Presence of diosgenin and absence of solasodine in tissue cultures of *Solanum laciniatum*. *Phytochemistry* 10: 1389-1390.

VAN DER MARK,F., PIJNACKER-HORDIJK,J.P., VARGA,G.A.I., DE VRIES,D.P. and DONS,J.J.M. 1990. *In vivo* transformation of clonal *Rosa canina* rootstocks with *Agrobacterium rhizogenes*. *Journal of Genetics and Breeding* 44: 263-268.

VANĚK,T., MACEK,T., STRÁNSKÝ,K. and UBIK,K. 1989. Plant cells immobilized in pectate gel: Biotransformation of verbenol isomers by *Solanum aviculare* free and immobilized cells. *Biotechnology Techniques* 3: 411-414.

VAN GELDER,W.M.J., VINKE,J.H. and SCHEFFER,J.J.C. 1988. Steroidal glycoalkaloids in tubers and leaves of *Solanum* species used in potato breeding. *Euphytica* Suppl: 147-158.

VAN UDEN,W., PRAS,N., HOMAN,B. and MALINGRÉ,T.M. 1991. Improvement of the production of 5-methoxypodophyllotoxin using a new selected root culture of *Linum flavum* L. *Plant Cell, Tissue and Organ Culture* 27: 115-121.

VAN WORDRAGEN,M.F., OUWERKERK,P.B.F. and DONS,H.J.M. 1992. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in chrysanthemum. *Plant Cell, Tissue and Organ Culture* 30: 149-157.

VERPOORTE,R., MULDER-KRIEGER,T., WIJNSMA,R., VERZIJL,J.M. and SVENDSEN,A.B. 1984. HPLC analysis of alkaloids in extracts of callus cultures of *Cinchona* species. *Zeitschrift für Naturforschung* 39: 680-683.

VICKERY,M.L. and VICKERY,B. 1981. *Secondary Plant Metabolism*. London: Macmillan.

VISSER,R.G.F., JACOBSEN,G., WITHOLT,B. and FEENSTRA,W.J. 1989. Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*. *Theoretical and Applied Genetics* 78: 594-600.

VOGEL,H., JATISATIENR,A. and BAUER,R. 1990. Quantitative analysis of the glycoalkaloids solasonine and solamargine and their localization in the leaves of *Solanum laciniatum* Ait. *Angewandte Botanik* 64: 393-400.

VOHORA,S.B., KUMAR,I. and KHAN,M.S.Y. 1984. Effect of alkaloids of *Solanum melongena* on the central nervous system. *Journal of Ethnopharmacology* 11: 331-336.

WALLER,G.R. and DERMER,O.C. 1981. Enzymology of alkaloid metabolism in

plants and microorganisms. *In:* E.E.Conn (ed.). *The Biochemistry of Plants - A Comprehensive Treatise. Volume 7. Secondary Plant Products*. New York: Academic Press. 317-402.

WALTON,N.J., ROBINS,R.J., BELSHAW,N.J., PARR,A.J., HAMILL,J.D. and RHODES,M.J.C. 1988. Perturbation of alkaloid biosynthesis in transformed root cultures of solanaceous plants by metabolic precursors and their analogues. *In*: R.J.Robins and M.J.C.Rhodes (eds). *Manipulating Secondary Metabolism in Culture*. Cambridge: Cambridge University Press. 73-78.

WEI,Z.-M., KAMADA,H. and HARADA,H. 1986. Transformation of *Solanum nigrum* L. protoplasts by *Agrobacterium rhizogenes*. *Plant Cell Reports* 5: 93-96.

WEILER,E.W., KRÜGER,H. and ZENK,M.H. 1980. Radioimmunoassay for the determination of the steroidal alkaloid solasodine and related compounds in living plants and herbarium specimens. *Planta Medica* 39: 112-124.

WELLS,M.J., BALSINHAS,A.A., JOFFE,H., ENGELBRECHT,V.M., HARDING,G. and STIRTON,C.H. 1986. *A Catalogue of Problem Plants in Southern Africa*. Pretoria: Department of Agriculture and Water Supply.

WHITE,P.R. 1963. *The cultivation of Animal and Plant Cells*. New York: The Ronald Press.

WHITNEY,P.J. 1992. Novel bioreactors for the growth of roots transformed by *Agrobacterium rhizogenes*. *Enzyme and Microbial Technology* 14: 13-17.

WIDHOLM,J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology* 47: 189-194.

WILLIAMS,D.H., STONE,M.J., HAUCK,P.R. and RAHMAN,S.K. 1989. Why are secondary metabolites (natural products) biosynthesized? *Journal of Natural Products* 52: 1189-1208.

YAHARA,S., MOROOKA,M., IKEDA,M., YAMASAKI,M. and NOHARA,T. 1986. Two new steroidal glucuronides from *Solanum lyratum*; II. *Planta Medica* 52: 496-498.

YAMADA,Y. and ENDO,T. 1984. Tropane alkaloid production in cultured cells of *Duboisia leichhardtii*. *Plant Cell Reports* 3: 186-188.

YANIV,Z., WEISSENBERG,M., PALEVITCH,D. and LEVY,A. 1981. Effect of seed number and fruit weight on content and localization of glycoalkaloids in *Solanum khasianum*. *Planta Medica* 42: 303-305.

YIN-LIN,Z. 1988. The transference of Ri-plasmid from *Agrobacterium rhizogenes* to *Scopolia lurida* revealed by the hairy root cultivation and the alkaloids production. *Acta Botanica Sinica* 30: 368-372.

YONEMITSU,H., SHIMOMURA,K., SATAKE,M., MOCHIDA,S., TANAKA,M., ENDO,T. and KAJI,A. 1990. Lobeline production by hairy root culture of *Lobelia inflata* L. *Plant Cell Reports* 9: 307-310.

YOSHIKAWA,T. and FURUYA,T. 1987. Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. *Plant Cell Reports* 6: 449-453.

ZEIGAN,D., VOIGT,G., HILLER K. and CUONG,N.X. 1987. ^{13}C-NMR-spektroskopische Charakterisierung eines 14β-hydroxylierten Steroidsapogenins aus *Dioscorea hispida* L. *Pharmazie* 42: 489-490.

ZWAYYED,S.K., FRAZIER,G.C. and DOUGALL,D.K. 1991. Growth and anthocyanin accumulation in carrot cell suspension cultures growing on fructose, glucose, or their mixtures. *Biotechnology Progress* 7: 288-290.

ZWEIG,G. and SHERMA,J. 1972. *CRC Handbook Series in Chromatography*. West Palm Beach: CRC Press.