INTRODUCTION
Phytochemicals are required from a spectrum of food for at least their antioxidant role, if not for other properties, to protect tissues from activities that manifest themselves into what we call chronic disease. Among the diverse groups of phytochemicals, phenolic antioxidants and antimicrobials from food plants are being targeted for designed dietary intervention to manage major oxidation-linked diseases such as diabetes, cardiovascular diseases, arthritis, cognition diseases and cancer. Foods containing phenolic phytochemicals are also being targeted to manage bacterial infections associated with chronic diseases such as peptic ulcer, urinary tract infections, dental caries and food-borne bacterial infections.
Introduction

Plant secondary metabolites are important sources of many food ingredients and disease-preventive phytochemicals. In recent years, the demand for these products has increased dramatically.

Plant phenolics are an important sub-group of secondary metabolites, which have diverse functional and medicinal applications. The emergence of dietary and medicinal applications for phenolic phytochemicals, harnessing their antioxidant and antimicrobial properties, in human health and wellness is not altogether surprising (Shetty and Wahlqvist, 2004).

Recent studies have indicated a role for phenolics from food plants in human health and, in particular cancer (Paganga et al, 1999). Phenolic phytochemicals have been associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Jorgensen et al, 1999). Phenolic phytochemicals (i.e. phenylpropanoids) serve as effective antioxidants due to their ability to donate hydrogens from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids and other biomolecules. Phenolic antioxidants, therefore, short-circuit a destructive chain reaction that ultimately degrades cellular membranes (Foti et al, 1994).

Plants produce phenolic metabolites as a part of growth, developmental and stress adaptation response. These stress and developmental responses are being harnessed to design consistent phytochemical profiles for safety and clinical relevancy using novel tissue culture and bioprocessing technologies (Shetty and Wahlqvist, 2004). The supply of the source plants for these secondary metabolites, however, is often limited because of disease, changes in climate, changes in the economical development or
other problems in the growing regions. Plant cell and tissue culture offers an alternative source for controlled production of these products (Tong-Jen Fu, 1998).

In the late 1970s plant cell culture was seen as an alternative or additional way of producing these compounds, since it was known that plant cells could be readily cultured and produce useful secondary metabolites (Alfermann and Peterson, 1995). Nevertheless, the low yields obtained with cultured cells, often inferior to the amounts present in intact plants, provided a major drawback to their commercial exploitation. Many strategies have been tried in attempts to increase product yield, including for instance the induction of differentiated cell cultures which are known to have a higher biochemical potential (Yeoman & Yeoman, 1996).

In the search for alternatives to production of desirable natural compounds from plants, biotechnological approaches, specifically, plant tissue culture has shown potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao and Ravishankar, 2002). Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. Discoveries of cell cultures capable of producing specific secondary metabolites at a rate similar or superior to that of intact plants have accelerated in the last few years. New physiologically active substances of medicinal interest have been found by bioassay. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating environmental factors, as well as by artificial selection or the induction of variant clones. Some of the important secondary metabolites localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by
undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformations of natural compounds has been demonstrated (Ravishankar and Rao, 2000). Due to these advances, research in the area of tissue culture technology for the production of plant chemicals has bloomed beyond expectations. The major advantages of a cell culture system over the conventional cultivation of whole plants according to Sajc et al. (2000) are:

1. Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions,

2. Cultured cells would be free of microbes and insects,

3. The cells of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites,

4. Automated control of cell growth and rational regulation of metabolite processes would reduce labor costs and improve productivity,

5. Organic substances are extractable from callus cultures.

Various food ingredients and phytochemicals, including flavors, colorants, essential oils, sweeteners, antioxidants, and nutraceuticals, have been produced in cell culture (Dornenburg and Knorr, 1996), some of which have been commercialized. For example, cultured ginseng products derived from cell suspension culture of *Panax ginseng* have been commercially produced by Nitto Denko Co. in Japan since 1990 with a net sale of $3 million in 1995 (Ushiyama, 1996). As more is known about the biochemical and genetic regulation of plant secondary metabolism, and more advances are made in the development of yield improvement strategies and design of
large-scale bioreactors, commercial development of PCTC-derived food ingredients is expected to increase (Tong-Jen Fu, 1998).

Several types of cell culture are used to produce food ingredients, including cell suspension, organized tissue, and transformed shoot and root cultures. Some characteristics associated with each of these systems are important to food safety (Tong-Jen Fu, 1998).

**Cell suspension cultures:** Cell suspension is the preferred type of culture for large-scale production because it is similar to microbial cultures and has rapid growth cycles. The existing microbial fermentation technology can be easily adapted to plant cell production. Plant cells are totipotent; that is, cells in culture can produce the same metabolites as the whole plant (Tong-Jen Fu, 1998). These are cell suspension cultures, organized tissue cultures and transformed tissue cultures.

**Organized tissue cultures:** Shoot, root, and other plant organ cultures have been developed for the production of compounds that require cell differentiation. The product profiles of these organized tissue cultures are similar to those of the field-grown plants (Endo and Yamaca, 1985). Genetic stability in these tissue cultures is much higher than in cell suspension or callus cultures. Stable growth and consistent secondary metabolite production have been observed in shoot and root cultures of many species (Charlwood and Moustou, 1988; Miura et al, 1988).

**Transformed tissue cultures:** Infection of wounded plants by *Agrobacterium rhizogenes* bacteria results in hairy-root disease, which is characterized by rapidly growing and highly branched roots at the host wound sites. T-DNA of the bacterial Ri (root-inducing) plasmid, which contains genes encoding endogenous hormones, is integrated into the plant genome and allows the proliferation of these adventitious
roots. Similarly, transformation of plant tissue with T-DNA from *A. tumefaciens* leads to the formation of adventitious shoots or "shooty teratomas". The use of hairy-root and shooty-teratoma cultures to produce food ingredients and other plant metabolites has attracted much attention. Many characteristics of these transformed tissue cultures are advantageous for plant metabolite production. First, these cultures grow faster than the untransformed roots or shoots, and in many cases, their growth rates approach those of cell suspension cultures (Flores et al 1987). Second, the pattern of secondary metabolite production in these cultures is similar to that in the parent plants (Parr and Hamill, 1987; Spencer et al 1990). Third, these cultures exhibit a high level of genetic (Aird et al 1988) and biochemical stability (Flores et al 1987).

In the present study, four different food materials were selected as these produce active ingredients with widespread application in the food industry, these being garlic, carrot, stevia and annatto plant whose active ingredients namely alliin, anthocyanin, stevioside and annatto, respectively, are either important food ingredients or provide specific nutritional benefits. Alliin is a precursor of allicin, a principal component of garlic which has medicinal as well as flavouring properties. Anthocyanin is a natural colouring agent and shows antioxidant activity. Stevioside is a natural non-caloric sweetener and with no side effects. It is used as an alternative sweetening agent. Annatto is a natural dye widely used as a natural colouring agent.

The review that follows is thus divided into four sections based on the four plant sources studied namely (1) garlic, (2) carrot, (3) stevia and (4) annatto.
Garlic (*Allium sativum;* Liliaceae/Alliaceae) is a perennial plant composed of a compound bulb consisting of individual bulbs, the latter generally known as cloves which are enclosed together in a white skin. Garlic has a long history of over 4,000 years of culinary and medicinal use. Research by Louis Pasteur in the 1800s documented that garlic kills bacteria. During World War-II, when penicillin and sulfa drugs were scarce, garlic was used as an antiseptic to disinfect open wounds and prevent gangrene. In the 1950s, Dr. Albert Schweitzer used garlic to treat cholera, typhus and amebic dysentery while he was working in Africa as a missionary. The Soviet army relied heavily on garlic during World Wars I and II where it earned the name “Russian penicillin”. During the great Plague epidemic, some herbalists avoided the deadly disease by eating large amounts of garlic and wearing garlic strands around their necks (Newall et al., 1996). To date, it has not been determined whether it’s antibiotic properties protected these people against the plague or whether the foul stench of the herb discouraged others from getting close enough to spread their infection (Milner, 2001).

In every 100 g of fresh weight, 23 g is carbohydrate accounting for the bulk of garlic bulb, apart from it’s rich content of proteins (4.4 g). Among the minerals, garlic is known to contain high levels of phosphorous (44 mg) followed by calcium (5 mg) and iron (0.4 mg). Vitamins like riboflavin (0.03 mg), thiamine (0.24 mg), nicotinic acid (0.9 mg), and vitamin-C are other important chemical constituents. In addition garlic also contains the minerals selenium and germanium. The amount of these minerals in the bulb depends on the content of the respective minerals in the soil where the bulb is grown. The essential oil, 0.2% in all, consists of allicin and many kinds of thio-ether...
compounds. Garlic also contains citral, geraniol, linalool, α-phellandrene, propionic aldehyde and valeraldehyde (Milner, 1996).

When garlic is crushed, it releases at least one hundred sulfur-containing compounds and it is sulfur that gives garlic its distinctive odor. Crushed garlic also releases an enzyme called alliinase and this enzyme converts alliin, the substance in raw garlic, to allicin (Bhagyalakshmi et al. 2000). Allicin is considered to be the most important of the biologically active components of crushed bulb. Allicin does not exist in garlic as such but is rapidly produced when the precursor alliin is cleaved by the action of alliinase upon crushing the tissue (Bhagyalakshmi, 1999).

Both alliin and alliinase are stable when dry and dried garlic still has the potential for releasing allicin when subsequently moistened (Lawson et al., 1991). Carefully processed high quality powder contains almost a similar chemical composition as that of freshly crushed garlic though oil-macerated powders appear to lose up to 80% of their sulphur compounds. Steam-distilled garlic oil is reported to contain dialk(en)yl sulphides (i.e., diallyl sulphide, allyl methyl sulphide, etc.,) as the major sulphur component ranging from 0.1% to 0.5%.

In garlic the predominant flavour precursor is alliin with lower concentrations of isoalliin and methiin and trace amounts of propiin (Lancaster and Shaw, 1991).
The allicin content in garlic leaf was observed to be increased slightly during maturation and increased rapidly in developing bulbs on maturation (Cho and Lee, 1974).

According the Unani and Ayurvedic systems as practiced in India, garlic is carminative and is a gastric stimulant and thus aids digestion and absorption of food. It is also given in flatulence. Allicin, a scavenger of peroxide radicals is responsible for the antioxidant activity of garlic bulb (Prasad et al, 1996).

The inhalation of garlic oil or garlic juice has generally been recommended by doctors in cases of pulmonary tuberculosis, rheumatism, sterility, impotency, cough and red eyes (Pruthi, 1979).

It has been indicated that garlic possesses insecticidal action. The repellent property of garlic has been demonstrated by Bhuyan et al (1974). A formulation containing 1% garlic extract gave protection to persons against mosquitoes and blackfly for about 8 hours. According to Sukul et al (1974) extracts of garlic showed strong nematicidal action of killing *Meloidogyne incognita* and other species of soil nematodes in less than 40 minutes. Similarly, in the studies of Debkirtaniya et al (1980), garlic extracts showed larvicidal properties with larvae of *Culex* species, *Spodoptera litura* and *Euproctis* species.

Sharma et al (1977) reported that a crude extract of garlic clove had antibacterial activity against gram positive and gram negative bacteria. According to Purseglove (1975), allicin of garlic has bactericidal properties. Barone and Tansey (1977) reported that there is inhibition of the growth of many zoo pathogenic fungi by aqueous garlic bulb extract. Use of garlic extract as seed treatment has been indicated by Russel and Mussa (1997) for the control of foot rot of French bean.
The enzyme alliinase [EC 4.4.1.4] in garlic tissues catalyses the conversion of alliin to the garlic flavour substance alliin, which has antibacterial and antifungal action (Cavallito and Baily, 1994). Alliin condenses to form ajoene which is antithrombotic in preventing the aggregation of blood platelets (Block et al, 1994). In addition, alliin itself enhances the effects of monosodium glutamate and disodium inosinate which improve the taste quality of food (Ueda et al, 1990). It is probably because of alliin that garlic has been valuable as an antibiotic, medicine and spice.

The hypocholesterolemic activity of garlic is mainly due to allicin, a principle compound of garlic. The raw garlic, garlic oil and allicin standardized garlic powder tablets showed significant cholesterol reduction (by 6-20%) in 6-16 weeks for a daily dose representing 2-3 garlic cloves (Lawson et al, 2001). Augusti (1997) reported that allicin, which has a hypocholesterolaemic action, is present in the aqueous extract of garlic and reduces the cholesterol concentration in human blood.

The concept of totipotency of individual cells formed the basis for the plant tissue and cell culture techniques which generated great interest in the propagation and manipulation of crop plants. Using tissue culture techniques, callus formation can be induced in numerous plant tissues and organs (Street, 1969).

Lee et al (1988) studied callus formation of garlic on Linsmair and Skoog medium. They observed that better callus formation was obtained from garlic stored at cold storage compared to room temperature. Callus proliferation and shoot production from callus culture were greatest in the presence of IAA at 2 mg l⁻¹ whereas IBA and BA had little effect.

Maggioni et al (1989) obtained optimum callus induction from leaf explant of garlic on MS medium supplemented with 2 mg l⁻¹ IAA, 0.5 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ Kn.
Shuto et al, 1993 obtained optimum callii formation from root apices of garlic on MS medium supplemented with 1.0 mg l\(^{-1}\) NAA in darkness for 2 weeks.

Kudou et al (1995) studied callus induction from different explants of garlic on MS medium supplemented with BA and NAA, each at 0, 1.0, or 2.0 mg l\(^{-1}\) in all combinations. The shoot tips, bulbs, basal plate and the lower, middle and upper part of foliage leaves were used as explants. Callus formation occurred for all types of explants and was promoted by increasing BA and NAA concentration.

Callus was induced from shoot tip, primordial as well as bulb leaf base. Explants of garlic were cultured on modified MS medium supplemented with various amounts of 2, 4, D, IAA and kinetin in darkness or 16 hr day period (about 2500 lux) at 23±2° C. Callus formation and growth were best on MS medium supplemented with 1.0 mg l\(^{-1}\) 2,4-D per liter in the dark (Moriconi, et al 1989).

Callus induction was observed from *in vitro* meristems, fragments of stem and meristematic root after inoculation on MS medium supplemented with 2,4-D. The presence of light had a positive effect on the induction percentage and size of calli. Under light conditions, the effect of 2,4-D concentrations was genotype dependant (Tapia, 1996).

Xue et al (1991) reported that embryogenic callus was induced from a receptacle on a BDS (Dunstan and Short) medium supplemented with 1 to 10 μM NAA. BA inhibited induction of embryogenic callus.

Intact garlic plants contain three flavor precursors s-allylcysteine sulphoxide, s-propylcysteine sulphoxide and s-methylcysteine sulphoxide. Undifferentiated white callus contain only s- methylcysteine sulpxioxide which does not have garlic flavour.
Full flavour precursor expression was found in green undifferentiated callus, redifferentiated green roots and redifferentiated green shoots. It is suggested that the biosynthetic pathway forming s-allyl and s-propylcysteine sulphoxides require the presence of differentiated plastids or optimally, chloroplasts (Lancaster et al, 1988).

In garlic, alliin is converted by alliinase to the flavour compound allicin, which has antibacterial, antifungal and various medicinal properties. When undifferentiated shoot or root callus tissues were analyzed, only shoot forming callus tissue contained alliin, suggested that alliin may be synthesized in the leaves (Hayashi et al, 1993).

Analysis of differentiated (with shoots) and undifferentiated bulb tissue culture showed that the contents of the flavour precursor alliin was higher in the differentiated cultures, accompanied by a greatly increased level of phenylalanine ammonia lyase (EC 4.1.1.5) activity (Mualpathak and David, 1986).

Addition of s-allyl-L-cysteine in the medium increased the levels of alliin (s-allyl-L-sulphoxide) in both root forming and shoot forming callus tissue of A. sativum (initiated from meristem tissue isolated from cloves). The oxidation of s-allyl-L-cysteine to alliin was examined using s-allyl-L[35s] cysteine and s-allyl-L[14C]cysteine and subsequent analysis of enzymatic degradation and of the isomer formation of product. Only (+) sulphoxide was isolated from the cells (-) s-allyl-L-cysteine sulphoxide was not formed in the cells. These findings suggested the presence of specific oxidase in the cells. The s-allyl-L-cysteine was quantitatively converted into alliin in this tissue during culture (Ohsumi et al, 1993).

Addition of allylmercaptan increase the formation of s-allyl-L-cysteine and alliin, which suggested that s-allyl-L-cysteine was formed from allylmercaptan and then converted to it's sulphoxide alliin. The s-allyl-L-cysteine substantially enhanced
Introduction

alliin formation within a few hours in both shoot forming and root forming callus tissue and maintained a high level during growth but the formation of alliin was lower in undifferentiated tissue (Ohsuri et al, 1993).

(2) CARROT:

*Daucus carota* var *sativus* (carrot) is a herbaceous biennial plant grown as an annual. In the first year, seedlings emerge with two strap-like leaves which are the cotyledons, followed by rosettes of doubly compound leaves arising from the crown. From the hypocotyl, a tap root develops. Carrots are grown for their prominent root structure and characteristic flavor and color. Carrots are grown as a cool season vegetable. Production of the enlarged hypocotyl occurs most significantly when cool nights slow the plant respiration, allowing for an accumulation of carbohydrates. Of all root crops, *D. carota* is considered the most important. High vitamin A, mineral and dietary fiber content of carrots contributes to their importance as a major food crop utilized in our diet.

Originally from Middle Asia, *D. carota* spread east and west, being cultivated in Europe as early as 1000 BC where it was grown as a medicinal plant, used to treat stomach problems, wounds, ulcers, liver and kidney ailments. Production as a food crop began in 600 AD in the region now known as Afghanistan. The first cultivated carrots were large and woody textured with a purple color. Yellow types were selected and cultured in Syria and Turkey in the 10th century; then spread to China in the late 13th century and to Europe in the 14th century.

Carrots constitute a valuable source of health-promoting ingredients such as carotenes and thus are important in human nutrition, with an annual world production of 21 Million tons in 2002 (FAOSTAT). Due to aggravated legal restrictions and the
Introduction

consumer's demand for natural food, synthetic additives and especially pigments are increasingly replaced by colorings originating from plants, such as anthocyanins. Nowadays, black carrots are enjoying increasing popularity as a source of natural food colorants (Giust and Wrolstad, 2003).

Healthy eating guidelines have directed the general public to eat more fresh fruit and vegetables throughout the world. Among these, carrots are being increasingly consumed, mainly due to their pleasant flavor and their perceived health benefits related to their vitamins, minerals, and fiber. Carrots have been ranked tenth in terms of nutritional value among 38 other fruits and vegetables, and seventh for their contribution to nutrition (U.S. Agricultural Statistics, 1971). Carrots have a complex flavor. There is no single compound that accounts for a distinctively carrot like flavor (Simon, 1985). There are many factors that influence carrot flavor, including nonvolatile chemical constituents such as free sugars, phosphates, and nitrogenous compounds (Alabran, and Mabrouk, 1973), bitter compounds (Carlton, 1961), phenolics (Sarkar and Phan 1979) and organic acids (Howard et al, 1995). The characteristic flavor of carrots s mainly due to the volatile constituents, which are mostly made up of terpenes and sesquiterpenes (Alasalvar, 1999).

Carrots are the major single sources of provitamin A, providing 17% of the total vitamin A consumption (Block, 1994). In carrots, six types of carotenes and related compounds exist, α-, β-, γ-, and δ-carotenes, lycopene, and β-zeacarotene. Recently, the demand for α-carotene has increased due to its reported anticancer activity in certain cases (Ziegler,1989) and other health benefits (Bendich, 1993; Baker and Meydany, 1994).
Introduction

The greatest number of embryos was obtained using young carrot callus cultured on Gamborg's B-5 medium containing 1 g l⁻¹ casein hydrolysate and 10⁻¹⁰ M 2,4-dichlorophenoxyacetic acid (2,4-D). The largest increase in embryogenesis for old cultures also was obtained using Gamborg's B-5 medium supplemented with 1 g l⁻¹ casein hydrolysate and 10⁻¹⁰ M 2,4-dichlorophenoxyacetic acid; however, no combination of factors for the older culture restored the initial vigorous morphogenic response seen in young cultures (Smith et al, 1997).

Narayan et al (2005) studied the influence of different growth regulators on biomass accumulation and anthocyanin content in solid-state and liquid-state batch cultures of *Daucus carota*. They reported that auxins such as 2,4-D, IAA and NAA supplemented at different levels, supported growth as well as AC synthesis, the maximum productivity of anthocyanin (1.27 g l⁻¹) was observed in the presence of 2.5 mg l⁻¹ of IAA followed by 1 mg l⁻¹ of NAA (0.5 g l⁻¹ of anthocyanin). Among the cytokinins, kinetin (0.1 and 0.2 mg l⁻¹) supported the highest anthocyanin productivity. The interplay of different levels of IAA and kinetin revealed that the combination of IAA at 2.5 mg l⁻¹ and kinetin at 0.2 mg l⁻¹ was superior to other combinations both in solid-state as well as liquid-state cultures, where anthocyanin productivity in solid-state was much higher (five-fold) than that in liquid-state. Long term effects of the best IAA and NAA levels indicated that only IAA could support uniform productivity of anthocyanin in solid-state only. In liquid-state cultures, anthocyanin synthesis dropped steadily up to six subcultures where the subsequent increase of IAA by 0.5 mg l⁻¹ brought back the level of anthocyanin for a limited number of subsequent subcultures. Increase of cytokinin did not improve anthocyanin productivity. Temperature also imparted significant effect on anthocyanin
productivity with 30 °C being ideal for solid-state whereas for liquid-state a temperature of 25 °C was the most suitable.

Narayan and Venkataraman (2000) studied the characterisation of anthocyanins derived from carrot (Daucus carota) cell culture. Two anthocyanin pigments were isolated from cell cultures of carrot. After chemical hydrolysis, column and paper chromatography, HPLC, proton and $^{13}$C NMR and mass spectroscopic studies indicated the presence of cyanidin-3-lathyroside [cyanidin-3-O{-D-xylopyranosyl (1→2) D-galactopyranoside}] (90%) and cyanidin-3-D-glucopyranoside (10%) in the callus cultures, whereas only cyanidin-3-lathyroside (0.05%) was found in the explant of carrot.

A high rate of embryo formation was observed in a suspension culture of a carrot callus when auxin was removed from the culture medium. Embryogenesis was inhibited by exogenously supplied 2,4-dichlorophenoxyacetic acid (2,4-D) ($> 10^{-9}$M) or indoleacetic acid (IAA) ($> 10^{-10}$M). Zeatin promoted the embryogenesis at a concentration of $10^{-7}$M. In contrast, other cytokinins such as benzylaminopurine (BAP) or kinetin did not promote but instead inhibited embryogenesis. Zeatin with auxins, 2,4-D or indoleacetic acid, did not show promotion but inhibition. Gibberellin A$_3$ (GA$_3$) or abscisic acid (ABA) did not affect the number of embryos formed in the globular and early heart stages but caused a decrease of those in the heart and torpedo stages (Fujimura and Komamine, 1975).

Edelman and Hanson (1971) reported that the substrate levels of sucrose were shown to reduce chlorophyll synthesis in carrot tissue culture in the strain CRT1 but not in the strain CRT2. In CRT1 the effect was shown to be a suppression of greening specifically by sucrose rather than a reducing sugar requirement for chlorophyll
Introduction

synthesis. In CRT1 sucrose caused both a reduction in chloroplast numbers per cell and a suppression of lamellar development in plastids. This effect on chloroplast structure was consistent with the observed reduced photosynthetic efficiency (micromoles CO$_2$ per hour per mg chlorophyll) of CRT1 calluses grown on sucrose.

Phenolic compounds in fruits and vegetables are of great interest in two respects. First, they contribute to the sensory qualities of fruits and vegetables: color, astringency, bitterness, and aroma. Second, some phenolics possess pharmacological properties and are used for therapeutic purposes (Macheix et al, 1990).

Surles et al (2004) analysed five different colored carrots for their carotenoid profile and conducted sensory evaluation to determine consumer acceptance ($n=96$). High $\alpha$-carotene orange carrots were found to contain the greatest concentration of total carotenoids. Except for the white, all the carrots are a significant source of bioavailable carotenoids. Sensory evaluation showed the high $\alpha$-carotene orange and white carrots to be favored over the yellow, red, and purple carrots in both blind and nonblind treatments ($p<0.01$). All the carrots were well accepted by the consumer panel. They suggested that carrot growers should be encouraged to cultivate specialty carrots to provide sources of both vitamin A precursors and phytochemicals.

Arabshahi et al (in press) studied the ethanolic extracts of drumstick leaves ($Moringa oleifera$), mint leaves ($Mentha spicata$) and carrot tuber ($Daucus carota$) for their antioxidant activity. They found that the extracts from drumstick and carrot had a higher antioxidant activity (83% and 80%) than $\alpha$-tocopherol (72%). In sunflower oil, the extracts from drumstick leaves and mint leaves were found to exhibit a similar activity (46% and 44%, respectively). The extract from drumstick exhibited the highest activity in both lipid systems. The antioxidant activity of the extracts from
mint leaves and carrot was higher at pH 9 than pH 4, while that of drumstick extract remained the same under both pH conditions. The extract from carrot was more heat-stable than other extracts. The three extracts stored in the dark at 5 and 25 °C after a 15 day period did not show any significant change ($p \leq 0.05$) in their antioxidant activity.

Nicole et al (2003) studied the effect of carrot intake on the antioxidant status in cholesterol-fed rats. Carrot consumption improved the antioxidant status. It significantly decreased the urinary excretion of thiobarbituric acid reactive substances (TBARS), reduced the TBARS levels in the heart, it increased the vitamin E plasmatic level and tended to increase the ferric reducing ability of plasma (FRAP) as compared to the control group.

Alasalvar et al (2001) studied four different colored carrots, orange, purple with orange core, yellow, and white, for their content of phenolics, antioxidant vitamins, and sugars as well as their volatiles and sensory responses. A total of 35 volatiles were identified in all carrots, 27 positively. White carrot contained the highest content of volatiles, followed by orange, purple, and yellow. In total, 11, 16, 10, and 9 phenolic compounds were determined for the first time in orange, purple, yellow, and white carrots, respectively. Chlorogenic acid was the most predominant phenolic compound in all carrot varieties. Differences ($p<0.05$) in relative sweetness, the contents of vitamin C, $\alpha$-and $\beta$-carotenes, and certain flavor characteristics were observed among the colored carrot varieties examined. Purple carrots contained 2.2 and 2.3 times more $\alpha$-and $\beta$-carotenes (trace in yellow; not detected in white) than orange carrots, respectively.
Stevia rebaudiana (Bertoni) was 'rediscovered' by Europeans in Paraguay in 1888 by Dr. M.S. Bertoni. He later botanically described and named the plant (in 1905) in honour of Paraguayan chemist Dr Rebaudi. Stevia is one of the 950 genera of the Compositae (Asteraceae). There are now known to be more than 150 Stevia species but this is the only one with significant sweetening properties; other species do contain other biochemicals of interest. It is native to a relatively small area of eastern Paraguay (on the Brazilian border) where its leaves have been used by the local Guarani Indians as a sweetener for many hundreds of years. They especially use it in the local green tea ("Mate" tea – Ilex sp.), as well as with otherwise unpalatable medicinal and other drinks. In its native state it is a perennial herb, living 3–5 years, with variable appearance up to about 0.7 meters tall. Stevia rebaudiana has been taken to many countries since it was first described by Bertoni and has subsequently been grown in latitudes well north of its native Tropic of Capricorn latitude. Mechanized agricultural production systems have not yet been developed and early initiatives to develop a sweetener industry based on Stevia usually lapsed as sugar became readily available (e.g. in U.K. during WWII). Interest has been rekindled in more recent years, especially in the developed world where diet conscious consumers seek a natural low-calorie sweetener as an alternative to chemical sweeteners. Chemical sweeteners such as aspartame are banned in Japan (Strauss, 1995). Stevia products are used commercially, extensively in Japan, using locally grown and imported (mainly from China) dried stevia leaves, where (at over 2,000 tonnes refined product) they make up over 40% of the non-sucrose sweeteners (the others being fructose, syrups, honey, etc) and 5–6% of the total sweetener market. In most other countries where it is used it is mainly used directly by consumers, rather than
Introduction

commercially. Domestic consumers add dried leaves, liquid extracts, crystals or powders to their drinks and cooking as a ‘herbal’ supplement (Shaffert, 1994).

_Stevia rebaudiana_ Bertoni is a perennial shrub of the Asteraceae (Compositae) family. Stevia is cultivated in many countries such as Paraguay, USA, Mexico, Central America, Japan, China, Malaysia, South Korea, Spain, Italy, Belgium and UK.

The main sweet component in the leaves of _Stevia rebaudiana_ Bertoni is stevioside (Figure 1). It’s content varies between 4 to 20% of the dry weight of the leaves. Melting point of stevioside is reported to be 197°C. It is highly soluble in ethanol and methanol, soluble in water, and stable in neutral or acidic aqueous solutions at 60°C or 100°C (Moussa et al, 2003). Other compounds present but in much lower concentration are: Dulcoside A, steviolbioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D and rebaudioside E (Geuns, 2002).

![Figure 1 Chemical structure of stevioside](image)

The established uses for Stevia products cover all those of artificial low-calorie (non-sucrose) sweeteners, aspartame etc. (Ahmad and Wong, 1985), as well as most other
purposes for which sugar can be used. The primary use is as a sweetener to enhance the palatability of foods and drinks. Unlike aspartame, Stevia sweeteners are heat stable up to 200°C, are acid stable and do not ferment, making them suitable for use in a wide range of products including baked/cooked foods. In some food uses it's lack of bulk makes it unsuitable to replace all of the sugar in recipes, such as confectioneries, icings etc. In addition to sweetening foods stevia extracts can increase the palatability and attractiveness of food through enhancement of flavours and odours (Ikan, 1993). Stevia products also have beneficial uses as herbal and medicinal products and for some more unusual uses, e.g. in tobacco products (Midmore and Rank, 2002).

Use of stevioside and stevia leaf extract:

Food and Culinary Uses

- Table top sweetener – for tea, coffee etc
- Soft drinks, cordials, fruit juices
- Ice-creams, yoghurts, sherbets
- Cakes, biscuits
- Pastries, pies, baking
- Jams, sauces, pickles
- Jellies, desserts
- Chewing gum
- Candies, confectioneries
- Sea-foods, vegetables
- Weight-watcher diets
- Diabetic diets
- Flavour, colour and odour enhancers
Introduction

A source of antioxidants
Alcoholic beverage enhancer (aging agent and catalyst)

Medicinal Uses

Toothpaste, mouthwashes – plaque retardant/caries preventor
Skin care – eczema and acne control, rapid healing agent
Diabetic foods and weight loss programs
Hypertension treatment and blood pressure control
Calcium antagonist
Bactericidal agent

Pill and capsule additive to improve taste

Other Uses

Tobacco additive and flavourant
Production of plant growth regulators (potential use)

Tissue culture:

Somatic embryos were obtained from floret explants of *S. rebaudiana* cultured on Murashige and Skoog medium supplemented with 2,4-D (9.05 or 18.10 μM) and kinetin (0-9.29 μM). On 9.05 μM 2,4-D supplemented medium without kinetin, maximum embryogenic callus formation occurred. On 18.10 μM 2,4-D supplemented medium, the best results were obtained with 2.32 micro M kinetin. Callus formation started at the base of the corolla and ovaries. Histological sections showed a fibrillar network on the surface of somatic proembryos. A unicellular origin for the somatic embryos is proposed (Filho and Hattori, 1997).

Somatic embryos were induced when leaves were cultured *in vitro* on MS medium supplemented with 2,4-D (10 or 25 μM) and BA (1 μM) and a high sucrose concentration (120 g/l). The embryos appeared to be formed directly without
Introduction

intermediate callus development. Somatic embryos failed to mature and developed roots but not shoot when transferred to MS medium without growth regulators and with a low (30 g/l) concentration of sucrose (Filho et al, 1993).

Bondarev et al (1998) studied on the effects of growth regulators such as NAA, 2,4-D, kinetin and benzyladenine (BA) on the callusogenesis and growth of *Stevia rebaudiana* cultured cells. It was observed that on leaf blades the callus formed was 2 to 3 times larger than that on stem fragments. NAA was more effective for cell culture growth than 2,4-D, while BA exhibited a stronger effect than kinetin. A significant retardation of callus growth and complete inhibition of organogenesis took place at 2,4-D concentrations ranging from 0.2 to 1.0 mg/l, whereas NAA at similar concentrations markedly stimulated callus growth. The rate of primary callus growth on leaf and stem explants was the highest at NAA to BA ratios of 2.0 mg/l:2.0 mg/l and 4.0 mg/l:1.0 mg/l, respectively. Analogous concentrations of these growth regulators were also found to be effective for further cell subculturing. The combination of NAA and BA was most suitable for the induction of shoot formation by *S. rebaudiana* explants, although the optimal concentration of these compounds was tenfold lower (0.1-0.2 mg/l). The addition of gibberellic acid to callus and suspension cultures resulted in a significant increase in their fresh weight due to cell expansion and hydration.

Bondarev et al (2001) studied a comparative analysis of production of steviol glycosides in intact plants, *in vitro* plants, dedifferentiated callus and suspension cultures, morphogenic callus and *in vitro* regenerated shoots. They found that the qualitative composition of the steviol glycosides in *in vitro* plants was found to be identical to that of intact plants, but their content in the former plants appeared to be about five or six times lower. A significant decrease in this value was not observed
Introduction

upon long-term cultivation (for about 5 years) of the plants. Non-differentiated cell cultures, such as callus and cell suspension, were shown to synthesize only minor amounts of the steviol glycosides, and their content varied greatly during the growth cycle of the culture. Qualitative composition of the steviol glycosides in the cell cultures appeared to be highly scant as compared with that of the donor plants. No correlation between the steviol glycoside content in organs of the donor plants and that in the cell cultures obtained was found. Factors determining plant cultivation conditions and influencing the accumulation of both, fresh and dry cell biomass were not able to completely induce the steviol glycoside synthesis in non-differentiated cell cultures.

Bondarev et al (2003) investigated the effects of sugars, mineral salts and plant growth regulators on the development of *Stevia* shoots cultivated in the roller bioreactor and their production of steviol glycosides. They concluded that the supplementation of fructose or glucose shows an extension of the shoots and development of their root system were much better than in the medium supplemented with sucrose. Under these conditions, accumulation of dry mass in the leaves decreased, and the content of steviol glycosides in leaves declined. At elevated sucrose concentrations (from 1 to 5%), enhanced development of the root system and an increase in plant dry mass and number of leaf pairs was observed. At the same time, 3% sucrose gave optimal steviol glycoside accumulation. A twofold elevation of the concentration of mineral salts considerably stimulated growth of the shoots, whereas the content of the steviol glycosides in their leaves decreased by about order of magnitude. Addition of 0.1 mg l\(^{-1}\) BA together with \(\alpha\)-naphthaleneacetic acid resulted in a 1.5-fold increase in the number of shoots. The shoots grown on BA-supplied medium displayed a strong inhibition of the development of their root
system. When the medium was supplied with gibberellic acid, lengthening of shoots and roots of *Stevia* were observed. All the plant growth regulators used strongly inhibited production of steviol glycosides.

Sikach (1998) studied the effect of nutrient medium components (mineral salts, vitamins, plant growth regulators, sucrose) on the growth of *Stevia rebaudiana* plants *in vitro*. He reported that the composition of the nutrient medium influenced plant growth rate, dry substance content, use of N, P and K from the medium, and the contents of diterpene glycosides in plants. The quality (content of diterpene glycosides) of *Stevia rebaudiana* can be regulated by changes in the composition of the nutrient medium.

Morini et al (2003) conducted studies on the *in vitro* propagation of different genotype of *Stevia rebaudiana*. Four genotypes, differing in some of their bioagronomic characteristics, were tested on MS culture medium, comparing the effects of two cytokinins: kinetin and 6-benzylaminopurine. Growth chamber temperature was 24±1°C, photoperiod was 16-h and light intensity was 45 plus or minus 5 micro mol m⁻² s⁻¹. Shoot rooting response was evaluated by IBA at 0.5 mg l⁻¹ and 0.1 mg l⁻¹. Susceptibility to tissue vitrification, very small leaves and thin and etiolated stem apical portions were associated particularly with 6-benzylaminopurine, in most genotypes. With kinetin, symptoms were attenuated but did not disappear completely. Among the genotypes tested, shoot proliferation ranged from about 1:4 to 1:9 and rooting from 65 to 92%. Fairly severe problems were encountered in plantlet acclimatization, with highest survival values not exceeding 80%. Micropropagation efficiency did not appear to be very satisfactory.
Shoot apex, nodal, and leaf explants of *Stevia rebaudiana* can regenerate shoots when cultured on MS medium supplemented with benzyladenine (BA; 8.87 μM) and IAA (5.71 μM). Rooting of the *in vitro*-derived shoots was achieved following subculture onto auxin-containing medium. A survival rate of 70% was recorded at the hardening phase on coco peat substrate. The presence of the sweet diterpene glycosides, namely stevioside and rebaudioside, was confirmed in the *in vitro*-derived tissues of *Stevia* using HPTLC techniques. Callus cultured on agar-solidified MS medium supplemented with BA (8.87 μM) and IBA (9.80 μM) showed the highest sweetener content (Sivaram and Mukundan, 2003).

A multiple shoot culture was induced from nodal segments of *Stevia rebaudiana* on MS (Murashige and Skoog) medium containing half-strength macroelements, 1% sucrose and supplemented with NAA (0.01 mg/l). A bioreactor with hormone-free MS medium (300 ml) was inoculated with 1.5 g of the multiple shoot culture and cultivated for a month. Culture of the multiple shoot culture in the bioreactor and transfer to *ex vitro* conditions took about 8-9 weeks. A total of approx equal to 600 plantlets were produced which could be transferred from greenhouse to field conditions (Nepovim and Vanek, 1998).

*In vitro* nodal segments of 6-week-old seedlings were cultured on MS medium with 50% macro element content and in the presence of 0.1 ppm NAA. At the transfer of plants from *in vitro* into *in vivo* conditions nodal segments were dipped in a 5% IAA solution to promote rooting. Treated plants were grown for 1 month in a greenhouse and then planted into the field. A most effective preparation for increasing the concentration of stevioside in leaves was Humiforte (synthetic amino acids, N, P, K and trace elements) in combination with Aminol (amino acids and N) but Melatran (lactic and anthranilic acids) gave the highest biomass yields (Acuna et al, 1997).
Stevia rebaudiana was micropropagated from shoot apex or intermodal explants on 3 different multiplication media and 2 rooting media. Shoot apices performed better as explants than nodal stem sections. Benzyladenine was more effective than kinetin in the multiplication medium. Roots emerged from the internodes on either of the rooting media (growth regulator-free or containing kinetin). Rooting was essential before plantlets were transferred \textit{ex vitro} (Constantinovici and Cachita-Cosma, 1997).

Cuttings were taken monthly over 12 months from apices of vegetative shoots on adult \textit{Stevia rebaudiana} plants. The 8-cm-long cuttings were partly defoliated and kept in the dark for 24 h with the bases held in solutions of IBA (5 x 10^{-5}, 1 x 10^{-5} or 5 x 10^{-6} M), IAA (2 x 10^{-4}, 1 x 10^{-4} or 1 x 10^{-5} M) or NAA (3 x 10^{-4}, 6 x 10^{-5} or 6 x 10^{-6} M). Control cuttings were dipped in water. The cuttings were inserted in a 1:1 soil:vermiculite mixture and rooting was assessed after 14 days. Treatment with IBA promoted rooting and increased the number of roots produced in cuttings taken in August and September, whereas IAA and NAA did so only for cuttings taken in August. Percentage rooting was highest (80\%) in cuttings taken in August and treated with IBA at 5 x 10^{-5} M. The treatments had no effect on percentage cutting survival after transplanting (Carvalho and Zaidan, 1995).

Two-cm-long nodal segments were excised from adult \textit{S. re\`{b}audiana} plants and cultured for shoot proliferation on MS medium containing 6 levels of NH$_4$NO$_3$, vitamins, 11 $\mu$M BA, 3\% sucrose and 1\% agar. When shoots were about 5 cm long they were transferred for rooting to full- or half-strength MS medium containing NAA at 0.0 to 10.0 $\mu$M. Lowering the NH$_4$NO$_3$ concentration in the multiplication medium from the standard 20.60 mM to 5.15 mM increased the number of shoots produced per nodal segment to an average of 10.90. The standard MS concentration of NH$_4$NO$_3$ also induced toxicity symptoms. Decreasing the MS salt level in the rooting medium...
by half increased the number of roots/shoot, and at this level 1.0 and, particularly, 10 
µM NAA had a beneficial effect on root induction. The survival rate of rooted plants 
on transfer to potting medium was 95% (Filho et al, 1992).

A laboratory procedure was developed for the clonal micropropagation of 
*S. rebaudiana*, using stem segments with 2 axillary buds. Use of MS medium without 
growth regulators gave results comparable to those obtained with growth regulators 
(BA + NAA and kinetin + NAA). Accordingly, plain MS medium is recommended as 
being cheap and effective. IAA at 0.5 mg/l gave good results in activating 
rhizogenesis. No reduction in growth was observed with increasing number of 
subcultures *in vitro* (Kornilova and Kalashnikova, 1996).

A field experiment was conducted at Bangalore, Karnataka, India, during kharif 1995- 
96, to study the effect of length of stem cuttings (7.5 or 15 cm) and growth regulator 
application (1000 ppm of IBA, NAA or IBA + NAA) on vegetative propagation of 
stevia. After dipping in growth regulators the cuttings were planted (2/hill) in the field 
and evaluated after 25 and 45 days. The percentage sprouting and shoot growth of 
sprouted cuttings were significantly higher with 15-cm cuttings compared with 7.5-
cm cuttings. Pre-treatment of cuttings with IBA or NAA or their mixture caused 
injury to callus tissue and resulted in very poor sprouting relative to the control. The 
direct planting of stem cuttings in the field had limited success, percentage sprouting 
being <40% even for the best treatments (Chalapathi et al, 1999).

Stefanini and Rodrigues (1999) studied the effect of gibberellic acid (GA₃; 10, 20 or 
50 mg/l) on the growth of *Stevia rebaudiana*. Growth attributes (fresh weight of 
roots, stems, leaves and whole plants) were determined at 14 day intervals. They 
observed that the best growth (root, stem, leaf and whole plant fresh weight) was
observed in the third harvest in the GA$_3$ at 50 mg l$^{-1}$ treatment. No trends were observed in the 10 and 20 mg l$^{-1}$ GA$_3$ treatments. However, the best overall growth was exhibited by the control.

Three treatments were tested using Germitest paper as the substrate for seed germination: (a) *Stevia rebaudiana* seeds with the hairs removed were placed on the paper, (b) intact seeds were so positioned that they touched the paper at one point, and (c) only the seed hairs were allowed to touch the paper. In all tests the temperature was a constant 25° C. Germination was 84.5, 76.85 and 61.68% in (a), (b) and (c), respectively. Seeds in (a), (b) and (c) took 3.87, 4.43 and 5.91 days, respectively, to attain 63.21% of maximum seed germination (Carneiro and Guedes, 1992).

Seeds of *Stevia rebaudiana* harvested in the greenhouse were placed in batches of 200 on germitest paper at constant temperatures of 20°, 25° or 30° C. Germination percentage was recorded at 12-h intervals. Combination of this data with Weibull's model of cumulative distribution with 3 parameters showed that best performance occurred at 25°. At 25°, 63.2% of maximum germination (90.03%) occurred after 101.4 h. The performance of the adjusted model was close to linear at temperatures of 20° or 25°; at higher temperature instability and asymmetry of results occurred (Takahashi et al, 2001).

*Stevia rebaudiana* seeds harvested in the greenhouse were subjected to accelerated aging at 40° C for 12-48 h before germination on moist paper at 25° C. Seed germination characteristics were evaluated at 12-h intervals in relation to the parameters of the Weibull function of cumulative distribution. Accelerated aging for 12 h accelerated germination. Treatment for 24 h reduced percentage germination.
Results were variable for seeds subjected to longer periods of accelerated aging (Carneiro, 1995).

Stevioside content:

Stevioside accumulation in callus cultures derived from *Stevia rebaudiana* leaf explants was positively correlated with cell organization and greening of the cultures but negatively correlated with callus growth rate. Callus shoot formation was not essential for stevioside synthesis. Stevioside content was highest in slow-growing, compact, green calluses with or without shoot formation (5.78 and 5.37%, respectively). Cells of these calluses were highly vacuolated and contained fully developed chloroplasts with dense stromata and plastoglobuli. Microbodies containing crystal lattices were closely associated with chloroplasts. Plastids in yellow, compact callus cells, which accumulated less stevioside (2.13%) than green calluses, contained many starch grains with few dispersed lamellae. Stevioside content was lowest (0.96%) in fast-growing, loose, yellow calluses, in which cell plastids were structurally simple and contained few lamellae (Shaoping et al, 1998).

Leaf explants of *Stevia rebaudiana*, cut into 1-cm² sections after surface sterilization, were cultured on MS medium containing different growth substances, at 25°C and 2000 lux, with a 12-h photoperiod. Results showed that stevioside content in differentiated callus was higher than that in undifferentiated callus. The ability to synthesize stevioside in callus derived from the dedifferentiation of leaves decreased markedly. The stevioside content of leaves of plants derived from the differentiation of callus was twice that of plants cultured in the field. The content of stevioside was highest in callus cultured on medium supplemented with 1 ppm GA; supplementation with IAA or NAA was less effective (Chen and Li, 1993).
Bondarev et al (2004) estimated the steviol glycosides content in different organs of *Stevia rebaudiana*. They found the plant organs contained different amounts of the steviol glycosides, with leaves containing the highest steviol glycoside content followed by the flowers, stems, seeds and roots. The highest steviol glycoside content was found in the upper young actively growing shoot sections, whereas lower senescent shoot sections recorded the lowest amount.

The quantitative determination of stevioside was carried out on dried leaves from plants of 16 different plots, using a new analytical method based on an HPLC/ELSD (Evaporative Light Scattering Detector) coupled system. The average levels of stevioside varied from 43.6 to 98.9 g kg\(^{-1}\). The results indicate that environmental and agronomic factors have more influence on stevioside production than the growth habit (Tateo et al, 1999).

The quantitative determination of stevioside was carried out by HPLC on dried leaves of 13 morphologically different types of plants. The average levels of stevioside in dried leaves varied from 102.3 to 134.6 g kg\(^{-1}\); this variability, related to the ratio of the weight of leaves to that of the whole aerial part, provides data for associating growth habit with stevioside output (Tateo, 1998).

Bovanova et al (1998) used an HPLC method for the determination of sweet-tasting stevioside (STS) in leaves of *Stevia rebaudiana* and in some beverages (tea, orange juice). The pre-separation procedure consisted of extraction of STS from the plant material using boiling water and a solid-phase extraction (SPE). Recovery rates of SPE for the analyzed matrices was in the range 92.8-97.8% (for concentrations of STS of 105, 210 and 300 micro g/ml; Relative Standard Deviation (RSD) less than or equal to 3.3%).
Biosynthesis of steviol glycosides:

The ent-kaurene skeleton of chloroplast diterpene glycosides, which are produced in large quantities in the leaves of *S. rebaudiana*, is formed via the recently discovered 2-C-methyl-D-erythritol 4-phosphate pathway. The enzymes catalyzing the first two steps of this pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), were characterized. Using reverse transcriptase-PCR, the *dxs* and *dxr* cDNAs were cloned, which comprise ORFs of 2148 and 1422 nucleotides, respectively. The cDNA-derived amino acid sequences for DXS and DXR contain 716 and 474 residues, encoding polypeptides of approximately 76.6 and 51 kDa, respectively. DXS and DXR from *S. rebaudiana* both contain an N-terminal plastid targeting sequence and show high homology to other known plant DXS and DXR enzymes (Totte et al, 2003).

*Stevia rebaudiana* leaves accumulate a mixture of at least 8 different glycosides derived from the tetracyclic diterpene, steviol. These natural products have similar biosynthetic origins to those of gibberellic acid (GA). The initial steps leading to the formation of GA result from the 2-step cyclization of geranylgeranyl diphosphate (GGDP) to (-)-kaurene via the action of 2 terpene cyclases (-)-copalyl diphosphate synthase (CPS) and (-)-kaurene synthase (KS). Steviol biosynthesis probably uses the same mechanism although the genes and enzymes from *Stevia rebaudiana* that are involved in the cyclization of GGDP have not been characterized. Both the *CPS* and *KS* genes from *S. rebaudiana* were isolated and recombinant CPS and KS were catalytically active, suggesting that the *CPS* and *KS* genes participate in steviol biosynthesis. The genes coding for *CPS* and *KS* are usually present in single copies in most plant species and their expression is normally low and limited to rapidly growing tissues. The *KS* gene has been duplicated in the *S. rebaudiana* genome and
both the KS and CPS genes were highly expressed in mature leaves, a pattern opposite to that found with GA biosynthesis. This pattern may lead to temporal and spatial separation of GA and steviol biosynthesis and probably helps to prevent over-expression from interfering with normal GA metabolism (Richman et al, 1999).

To lay a foundation for molecular breeding efforts, the first genetic linkage map for Stevia rebaudiana has been constructed using segregation data from a pseudo test-cross F1 population. A total of 183 randomly amplified polymorphic DNA (RAPD) markers were analysed and assembled into 21 linkage groups covering a total distance of 1389 cM, with an average distance between markers of 7.6 cM. The 11 largest linkage groups consisted of 4-19 loci, ranged in length from 56 to 174 cM, and accounted for 75% of the total map distance. Fifteen RAPD loci were found to be unlinked. From the 521 primers showing amplification products, 185 (35.5%) produced a total of 293 polymorphic fragments, indicating a high level of genetic diversity in stevia. Most of the RAPD markers in stevia segregated in normal Mendelian fashion (Yao et al, 1999).

Foods Safety:

Suanarunsawat et al (2002) conducted an experiment to clarify the effect of Stevia rebaudiana (SR) on plasma glucose level (\(P_G\)) in streptozotocin-induced diabetic rats (STZ-induced diabetic rats). Four groups of rats were established including two groups of normal rats and two groups of diabetic rats. The first two groups of normal rats were fed daily with water or an aqueous extract of SR (4.66 g kg\(^{-1}\) body weight) for eight weeks. The other two groups of diabetic rats were treated in the same manner as normal rats. The results showed that there was no significant difference of the \(P_G\) in normal rats fed either with or without SR throughout eight weeks. \(P_G\) was
gradually raised in diabetic rats throughout the experimental period. Daily feeding of SR delayed the rise of \( P_C \) in diabetic rats fed SR (DM-SR). The \( P_C \) in DM-SR became significantly lower than that of normal diabetic rats (<0.05) since the third week until the end of the experiment. Daily feeding of SR had no effect on urinary glucose excretion in both normal and diabetic rats throughout the eight weeks. Feeding of SR had no effect on serum insulin and plasma glucagon concentrations in normal rats, whereas it potentiated insulin release from 2.66 ± 0.19 to 3.87 plus or minus 0.45 \( \mu \)IU/ml (\( P<0.05 \)) and suppressed glucagon level from 76.04 ± 5.38 to 49.43 ± 3.45 pg/ml (\( P<0.01 \)) in DM-SR. There were no significant changes of creatinine clearance and urinary protein excretion in both normal and diabetic rats fed with SR.

Both stevioside and steviol (1 nmol/l to 1 mmol/l) dose-dependently enhanced insulin secretion from incubated mouse islets in the presence of 16.7 mmol/l glucose (\( P<.05 \)). The insulinotropic effects of stevioside and steviol were critically dependent on the prevailing glucose concentration, ie, stevioside (1 mmol/l) and steviol (1 micro mol/l) only potentiated insulin secretion at or above 8.3 mmol/l glucose (\( P<.05 \)) (Jeppesen et al, 2000).

Suanarunsawat and Chaiyabutr (1997) studied the effect of stevioside (SVS; 150 mg/ml solution) on glucose metabolism in rats. SVS was administered either by intravenous infusion (hourly dose of 0.67, 1 or 1.33 ml/kg) or feeding (13.33 ml/kg). Plasma glucose level significantly increased both during and after SVS infusion; it was not affected by SVS feeding. The glucose turnover rates of \(^{14}\text{C}(U)\)glucose and \([3-\text{H}]\)glucose were not significantly different between control and SVS-infused animals, but percentage glucose carbon recycling and glucose clearance were reduced. Plasma insulin level did not change, whereas plasma glucose level significantly increased from 120.3 plus or minus 5.9 to 176.8 plus or minus 10.8 mg% during SVS.
infusion. SVS infusion had no significant effect on animals pretreated with angiotensin II or arginine vasopressin, but an attenuated hyperglycaemic effect was observed in animals pretreated with prazosin. Pretreatment with indometacin in or Nω-nitro-L-arginine methyl ester (L-NAME) alleviated plasma glucose level during the second period of SVS infusion. Pretreatment with indometacin – L-NAME reduced plasma glucose level from 117 plus or minus 1.8 to 109.0 plus or minus 1.7 mg%, and normalized plasma glucose level in the second period of SVS infusion. Insulin infusion inhibited the hyperglycemic effect of SVS infusion. The elevation of plasma glucose level during SVS infusion was not due to a reduction of insulin level, but could be due to an effect on glucose transport. Insulin response to a high plasma glucose level was suppressed during SVS infusion.

The methanolic extracts prepared from ten culinary herbs (*Salvia officinalis, Melissa officinalis, Aloysia triphylla, Origanum vulgare, Mentha piperita, M. spicata, Rosmarinus officinalis, Thymus vulgaris, Stevia rebaudiana* and *Ocimum basilicum*) were investigated for their cytotoxic effect against L1210 cancer cells and their mode of action. Substantial cytotoxic effects were observed in all cases, with the most prominent effect demonstrated by lemon verbena extract showing 87 plus or minus 4.1% cytotoxicity at 100 micro g/ml and 3 days culture period. The cytotoxic effect was dose- and culture period-dependent. With respect to the mechanism of the cytotoxicity, the augmented generation of $\text{O}_2^-$ ion and the dramatically escalated activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase with the addition of the herb methanol extracts suggested the involvement of reactive oxygen species metabolism in the course of L1210 cancer cell death by the methanol extract of the edible herbs (Kim et al, 2002).
Four steviol (ent-kaurene-type diterpenoid) glycosides viz., stevioside, rebaudiosides A and C, and dulcoside A, have been isolated from *Stevia rebaudiana*. These compounds showed strong inhibitory activity against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in 7-week-old female mice. The 50% inhibitory dose of these compounds for TPA-induced inflammation was 54.1-291.6 µg/ear. Furthermore, at 1.0 and 0.1 mg/mouse of stevioside mixture, the mixture of these compounds markedly inhibited the promoting effect of TPA (1 µg/mouse) on skin tumour formation initiated with 7,12-dimethylbenz[a]anthracene (50 µg/mouse) (Yasukawa et al, 2002).

Toyoda et al (1997) evaluated the carcinogenicity of stevioside in F344 rats. Stevioside was added to powdered diet at concentrations of 0 (control), 2.5 and 5%. The doses were selected on the basis of results from a 13-week subchronic toxicity study and administered to groups of 50 male and 50 female rats *ad lib*, for 104 weeks. All surviving rats were killed at week 108. Body weight gains were slightly depressed in line with the dose of stevioside, in both sexes, and a significant decrease in the final survival rate was observed for the 5% treated males. Histopathologically, however, there was no significantly altered development of neoplastic or non-neoplastic lesions attributable to the stevioside treatment in any organ or tissue, except for a decreased incidence of mammary adenomas in females and a reduced severity of chronic nephropathy in males.

Lee et al (2001) reported that intraperitoneal injection of stevioside 25 mg/kg also has antihypertensive effect in SHRs. In isolated aortic rings from normal rats, stevioside could dose-dependently relax the vasopressin-induced vasoconstriction in both the presence and absence of endothelium.
Chan et al (1998) studied the effect of intravenous stevioside on blood pressure in spontaneously hypertensive rats (SHR). The hypotensive effect on systolic and diastolic blood pressure was dose-dependent for intravenous doses of stevioside 50, 100 and 200 mg/kg in conscious SHR. The maximum reductions in systolic and diastolic blood pressure were 31.4 plus or minus 4.2 and 40.8 plus or minus 5.6%, respectively and the hypotensive effect lasted for more than 60 min with a dose of stevioside 200 mg/kg. Serum dopamine, norepinephrine and epinephrine levels were not changed significantly 60 min after intravenous injection of stevioside 100 mg/kg in anaesthetized SHR.

Steviol (0.5, 1.0 and 3.0 mg kg$^{-1}$ h$^{-1}$, i.v.) elicited no significant changes in mean arterial pressure (MAP), glomerular filtration rate (GFR) and renal effective plasma flow (ERPF). The steviol infusion (1.0 and 3.0 mg kg$^{-1}$ h$^{-1}$), however, induced a significant increase in the fractional sodium excretion (FeNa$^+$), fractional potassium excretion (FeK$^+$), and urinary flow as percent of glomerular filtration rate (V/GFR) when compared with controls. The data suggests that steviol may affect salt and water transport in renal tubules (Melis, 1997).

Toskulkao et al (1994) studied the relationships between urinary enzyme levels and changes in blood urea nitrogen (BUN) and plasma creatinine levels, along with simultaneous ultrastructural changes of the kidney, in rats treated with stevioside. BUN levels increased at 3 h onward after administration of stevioside (1.5 g/kg). The maximum increase in BUN and creatinine levels were approximately 180% and 132% at 9 h after stevioside injection, respectively. At this time, stevioside also caused significant increases in glucosuria, and alkaline phosphatase and $\gamma$-glutamyl transpeptidase [$\gamma$-glutamyltransferase] levels, but no significant changes in proteinuria, and N-acetyl-$\beta$-D-glucuronidase or glutathione-S-transferase [glutathione-
transferase] levels. Stevioside administration caused degeneration of proximal convoluted tubule cells in the kidney, but did not affect lipid peroxide levels. These results suggest that stevioside-induced nephrotoxicity involves the proximal convoluted tubules, and could be caused by a defect of cell volume regulation due to depletion of intracellular ATP and disruption of microvilli, and nuclear dysfunction.

The effects of oral administration of *Stevia rebaudiana* leaf extracts (plant material collected in Paraguay) for 20, 40 and 60 days on renal function and mean arterial pressure in normal Wistar rats were evaluated by Melis (1995). Results showed that rats treated with *S. rebaudiana* for 20 days did not significantly differ from the control group. Chronic administration of the crude extract for 40 or 60 days induced hypotension, diuresis and natriuresis but the glomerular filtration rate (GFR) remained constant. An increase of the renal plasma flow (RPF) was exclusively observed for the group treated for 60 days. The results suggest that oral administration to rats of an aqueous extract of dried leaves induces systemic and renal vasodilation, causing hypotension, diuresis and natriuresis.

Koyama et al (2003) conducted research on the *in vitro* metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Degradation was examined by incubating stevia mixture, enzymatically modified stevia, rebaudioside A, α-monoglucosylstevioside, α-monoglucosylrebaudioside A and the aglycone, steviol with pooled human faecal homogenates (obtained from five healthy volunteers) for 0, 8 and 24 h under anaerobic conditions. Stevia mixture, enzymatically modified stevia, stevioside and rebaudioside A (0.2 mg/ml) were completely eliminated within 24 h, whereas no degradation of steviol (0.08 and 0.2 mg/ml) appeared to be found during the
incubation period. Stevia mixture, stevioside and rebaudioside A appeared to be hydrolyzed to steviol by human intestinal microflora.

Stevioside orally administered to pigs was completely converted into steviol by the bacteria of the colon. No stevioside or steviol could be detected in the blood of the animals, not even after converting steviol into the (7-methoxycoumarin-4-yl)methyl ester of steviol, a very sensitive fluorescent derivative with a detection limit of about 50 pg. The intestinal transport characteristics of stevioside, rebaudioside A and steviol were also studied in the Caco-2 system. Only a minor fraction of stevioside and rebaudioside A was transported through the Caco-2 cell layer giving a $P_{app}$ value of $0.16 \times 10^{-6}$ and $0.11 \times 10^{-6}$ cm/s, respectively. The $P_{app}$ value for the absorptive transport of steviol was about $38.6 \times 10^{-6}$ cm/s while the $P_{app}$ value for the secretory transport of steviol was only about $5.32 \times 10^{-6}$ cm/s suggesting carrier-mediated transport. The discrepancy between the relatively high absorptive transport of steviol and the lack of steviol in the blood may be explained by the fact that in the Caco-2 study, steviol is applied as a solution facilitating the uptake, whereas in the colon steviol probably is adsorbed to the compounds present in the colon of which the contents is being concentrated by withdrawal of water (Geuns et al, 2003).

Takahashi et al (2001) carried out work on an anti-human rotavirus (HRV) activity of hot water extracts from *Stevia rebaudiana*. SE inhibited the replication of all 4 serotypes of HRV *in vitro*. This inhibitory effect of stevia extract was not reduced on the prior exposure of stevia extract to HCl for 30 minutes at pH 2. Binding assay with radiolabelled purified viruses indicated that the inhibitory mechanism of stevia extract is the blockade of virus binding. The stevia extract inhibited the binding of anti-VP7 monoclonal antibody to HRV-infected MA104 cells (African rhesus monkey kidney cells). The inhibitory components of stevia extract were found to be heterogeneous
Introduction

anionic polysaccharides with different ion charges. The component analyses suggested that the purified fraction named as stevia with the highest inhibitory activity consists of the anionic polysaccharide with molecular weight of 9800, and contains Ser and Ala as amino acids. Analyses of sugar residues suggest uronic acid(s) as sugar components. It did not contain amino and neutral sugars and sulfate residues.

Food Application:

The range of low-calorie sweeteners available to the food industry is expanding. It is essential to have an exact knowledge of the relative sweetness of various sweeteners in relation to different sucrose concentrations. Cardello et al (1999a) determined the variation on the relative sweetness of aspartame (APM), stevia (*Stevia rebaudiana*) leaf extract (SrB) and the cyclamate/saccharin mixture (two parts of cyclamate and one part of saccharin (C/S)) with the increase in their concentrations, and in neutral and acid pH in equi-sweet concentration to 10% sucrose, using magnitude estimation. Sweetness equivalence of SrB in relation to sucrose concentrations of 20% or higher and of APM and C/S to sucrose concentrations of 40% or higher could not be determined, because a bitter taste predominated. The potency of all sweeteners decreased as the level of sweetener increased. In equi-sweet concentration of sucrose at 10%, with pH 7.0 and pH 3.0, the potency was practically the same for all sweeteners evaluated.

Sweetness equivalence studies indicated that stevia leaf extracts were 152 times sweeter than 3% sucrose and 97 times sweeter than 10% sucrose. The time-intensity curves for sweetness of 3% and 10% sucrose and of stevia of equivalent sweetness indicated that the duration of the sweet stimulus was greater for stevia than for sucrose, particularly at 10% sucrose level where the sweet stimulus lasted for 32
seconds with sucrose and 96 seconds with stevia. Sucrose had no bitter taste whereas stevia left a bitter after taste, particularly at the 10% sucrose equivalent level (Cardello et al, 1999b).

Maia et al (2003) conducted research on formulations of low caloric drinks based on acerola (*Malpighia emarginata*). The drinks were prepared using 5 different sweeteners: acesulfame-K, aspartame, cyclamate, saccharine and stevia. The sweeteners were used alone or in combination, at concentrations not exceeding the limits set by the Brazilian food legislation. The final products were submitted to sensory evaluation, and their physicochemical and microbiological properties were assessed on days 0, 30, 60, 90 and 120 of storage at 25° C. No statistically significant variations were observed for pH, deg Brix, deg Brix:acidity ratio and reducing and non-reducing sugar contents. Ascorbic acid contents varied at different points of the storage period. No pathogenic or spoilage organisms were detected in the final product. The product containing a combination of aspartame, saccharine and sodium cyclamate was the most acceptable.

Stevioside crystals occurred in different forms and their sweetness equivalence was about 200 times more than that of 0.4% sucrose solution. No significant differences could be traced regarding taste (sweetness), flavour or acceptability among tea, anise, peppermint, lemonade and nescafe sweetened with stevia sweetener (100 mg/150 ml) or with sucrose (5000 mg/150 ml). Likewise biscuits, cake and yoghurt sweetened with 50% stevia sweetener and 50% sucrose resulted in the same sensory properties of 100% sucrose and, at the same time, reduced their caloric content (Moussa et al, 2003).
The sensory properties of aspartame, extract of stevia leaves, and a mixture of cyclamate and saccharin (2:1) in solutions at 10% sucrose equivalence were evaluated by Cardello et al (2003) for sweetness and bitterness using descriptive analysis and time-intensity analysis. In the descriptive analysis, the initial and residual sweetness, initial and residual bitterness, liquorice flavour, and body were evaluated using non-structured 9 cm scales. The parameters obtained from the time-intensity curves were maximum intensity, time taken to reach maximum intensity, time corresponding to the point where maximum intensity begins to decline, time of duration of maximum intensity, the area under the curve, and total time of duration of the stimulus. The results from the analyses indicated that aspartame is a sweetener with characteristics that most resemble sucrose and that stevia extract differed most.

Salem and Massoud (2003) studied the effect of stevia leaves powder as a natural non-caloric sweetener on the physico-chemical properties of fibre fortified frozen yoghurt. Frozen yoghurt mixes were prepared with different proportions of sucrose and stevia leaves powder. Addition of increasing proportion of stevia powder had considerable effect on total solids content but had a slight effect on the protein content of frozen yoghurt. Also it affected the specific gravity and freezing point of frozen yoghurt. The melting resistance of the product was improved by increasing the level of stevia sweetener added. Replacing sucrose with stevia sweetener reduced the caloric value of the frozen yoghurt by about 33.86%, and improved it’s flavour. Stevia sweetener powder can be used to replace 75% of sucrose which gave frozen yoghurt good texture and melting resistance.

Parpinello et al (2001) conducted research on the suitability of stevioside as a sweetener in peach juice. Comparison between stevioside and sucrose in terms of sweetness, sweet and bitter aftertastes were determined both in water and peach juice.
The results demonstrated that 160 mg/L of stevioside may replace 34 g/L of sucrose in juice, with a 25% decrease in calories, without affecting the sensory characteristics of the product. Synergistic and inhibitory effects between sucrose and stevioside were also monitored at different stevioside concentration. A new juice formulation sweetened with a binary mixture of stevioside (160 mg/L) and sucrose (56 g/L) was not significantly different in terms of desirability from a reference product sweetened with 9% sucrose.

(4) ANNATTO (BIXA ORELLANA):

Annatto (Bixa orellana L.) is a perennial shrub widely cultivated in tropical and subtropical regions of the world (Little et al, 1974). It’s single or multiple stems are light brown. The bark is more or less smooth with many warty lenticels, but may become fissured in old individuals. If given ample space, annatto generally branches several times near the ground and develops a dense, spreading crown. The plant roots firmly with a thick taproot and finer laterals. The alternate leaves have long petioles, thin ovate blades with long-pointed tips. Panicles at the branch tips have few to many pink or white flowers. The fruits are spiny capsules that dry and split open in two parts to expose red seeds on their inner surfaces (Howard 1989, Liogier 1995). Annatto is shade-intolerant and must have disturbance or a broken forest canopy to become established. All the naturally growing annatto shrubs in Puerto Rico are found on neglected or abandoned farmland, some of which has grown up to early secondary forest. Rainfall ranges from 1000 mm to 3000 mm per year in areas where the species grows naturally or under cultivation. Soils with textures from sands to clays are colonized. The species tolerates relatively low base saturation and moderate
compaction. Anatto is vulnerable to overtopping and smothering by trees, shrubs, vines, and grass. Plants that have become overtopped and shaded cease to flower and bear fruit. The species is frost sensitive (Carlowitz 1991).

Annatto shrubs will bear fruit when 2 years old in Hawaii (Neal 1965). Under good management, plants will fruit within 1 year of planting (Nepstad et al, 1991). An Indian plantation yielded 529 kg/ha of seed at 2 years old and 2,483 kg/ha of seed at 3 years old (Kanjilal and Singh, 1995). Annatto seldom reaches more than 5 m in height and 10 cm in stem diameter (Little et al, 1974). Pruning of ornamentals is recommended to shape and thicken the crowns (Warren 1997).

*Bixa orellana* accumulates a commercially important natural dye, constituted mainly of bixin and norbixin carotenoids, in the seed coat. This dye is used as a substitute to synthetic dyes in both food and cosmetic industries. One of the advantages of using natural dyes is that they are generally more widely accepted in foodstuffs rather than their synthetic counterparts (Henry, 1996).

Annatto, obtained from the oily arils of the seeds is the world’s second most important (after caramel) natural colorant (Mercadante and Pfander 1998), yielding yellow to red colors. The colors are produced by several apocarotenoides and may reach up to 7 percent of the seed’s dry mass (Katzer 1999). World production of annatto seed, both for commercial and home use, was estimated in 1990 at 10,000 tons per year (Arkcoll 1990). Brazil is the world’s largest exporter (Katzer 1999). Not only was the dye used anciently to color food, but also to dye cloth and paint the skin (which is still done today). The species is also planted as an ornamental, particularly the varieties with bright pink pods. Branches with the dry pods are used in dry floral arrangements (Warren 1997). Bees collect nectar from it’s flowers to make honey.
The wood is lightweight (specific gravity 0.4), weak, and not durable. It was used in 
former times to start fires by friction. Ropes and twine were made from the fibrous 
bark (Little et al, 1974). The pulp surrounding the seeds is widely used in herbal 
medicine to treat burns, bleeding, dysentery, gonorrhea, constipation, and fever 
(Parrotta, 2001). Extracts of leaves, bark, and roots are reported to be antidotes for 
poisoning from Manihot esculenta Crantz, Jatropha curcas L., and Hura crepitans L. 
(Liogier 1990).

Annatto extracts have been used for over two centuries as a food colour, especially in 
cheese, and various types are now used in a wide range of food products. Annatto 
extracts are obtained from the outer layer of the seeds of the tropical tree Bixa 
orellana. The principal pigment in annatto extract is cis-bixin, which is contained in 
the resinous coating of the seed itself. Processing primarily entails the removal of the 
pigment by abrasion of the seeds in an appropriate suspending agent. Traditionally, 
water or vegetable oil is used for this purpose, although solvent extraction is also 
employed to produce annatto extracts with a higher pigment content. Microcrystalline 
bixin products of 80–97% purity have been developed in response to the need for 
more concentrated annatto extracts (Kroes and Verger, 2004).

Neto et al (2003) studied the relative importance of explant, cytokinin type, carbon 
source and gelling agent for annatto organogenesis. The best organogenic response, 
including adventitious shoot number and elongation, was obtained when hypocotyl 
segments and rooted hypocotyls were cultured onto MS medium supplemented with 
4.56 µM zeatin, 87.6 mM sucrose, and 2.8 g l⁻¹ phytogel. Adventitious shoots derived 
from hypocotyl segments were less frequent and more difficult to elongate than those 
derived from rooted hypocotyls. Thidiazuron (TDZ) promoted a higher organogenic 
response in rooted hypocotyls, resembling a rosette-like structure, but impaired shoot
elongation. Histological investigation showed that zeatin-induced meristemoids originated mainly from wounding tissues, and that TDZ induced a high level of mitotic division resulting in several proliferation zones nearby the epidermis and outer cortical tissues. Rhizogenesis efficiency (rooting frequency and root number) was greater at the highest indole-3-butyric acid (IBA) concentration (5.0 μM) employed, although calli occurred at the basal end of shoots. Eighty percent of rooted plantlets survived after acclimatization.

A protocol has been developed for plantlet regeneration from seed callus of *Bixa orellana* L. Seeds demonstrated a high percentage of callus induction (63.8±7.3%) and a high yield (356.3±14.7 mg per seed) of white friable callus on Murashige and Skoog (MS) medium containing 5.0 μM 1-naphthaleneacetic acid (NAA) and 2.5 μM \( \text{N}^6 \)-benzyladenine (BA) within 6 weekk of culture in the dark. Callus induction frequency was greater under 24 h dark as compared to 16 h light/8 h dark photoperiod or 24 h light photoperiod. Increased myo-inositol (MI; 200 mg l\(^{-1}\)) and addition of ascorbic acid (AA; 200 mg l\(^{-1}\)) to the culture medium positively improved callus induction frequency and growth. Shoot differentiation from white friable seed callus was best using 10.0 μM BA and 5.0 μM NAA, where the highest percentage of calluses forming shoots (74.9±4.8%), the highest number of shoots per callus (six or seven) and the highest shoot-forming index (5.0) were obtained within 6 wk. Shoots elongated to 4 cm within 4 wk of transfer onto MS medium devoid of growth regulators. Shoots were rooted using half-strength MS medium containing 5.0 μM indole-3-butyric acid (IBA). About 85% of these plants were established in pcts containing pure garden soil and organic manure after 3 wk of hardening. Regenerated plants were morphologically uniform with normal leaf, shape and growth patterns. (Khan et al, 2002).
This review presented above was related to the four plant material investigated in this study namely garlic, carrot and annatto.

The objectives of the present study were multifold:

1. To optimize conditions for callus culture of the selected foodstuff namely garlic, carrot, stevia and annatto plant with a view to assess the presence of their active ingredients followed by their separation and quantification. This was attempted with a view to explore the possibility of the callus as a source of the active ingredient.

2. To attempt micropropagation of stevia plant containing high levels of stevioside from nodal explants followed by hardening since stevia plant produced by seed propagation shows widespread variation in stevioside content. Thus, to assess the use of tissue culture as a preferred medium for increasing the production of stevia plants containing optimum levels of stevioside.

3. To study stevia leaves for their nutrient composition, phytochemical and mineral content as well as fatty acid composition as there is very little information available on these lines.

4. To study the antimicrobial activity of stevia leaves as very few studies have been carried out on this aspect of stevia plant.

5. To study the application of stevia leaves and pure stevioside powder in various food preparations such as lemon juice, tea and coffee as an alternate sweetening agent.