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Review Article

FOOD, ETHANOBOTANICAL AND DIVERSIFIED APPLICATIONS OF *BIXA ORELLANA* L.: A SCOPE FOR ITS IMPROVEMENT THROUGH BIOTECHNOLOGICAL MEDIATION

Akshatha Venugopalan, *P. Giridhar and G. A. Ravishankar
Plant Cell Biotechnology Department, Central Food Technological Research Institute (A Constituent Laboratory of Council of Scientific and Industrial Research), Mysore- 570 020, India
**Author for Correspondence*

ABSTRACT
Bixa orellana L. popularly familiar as annatto plant belonging to *Bixa*ceae is known for reddish orange dye 'annatto' that is produced in aril portion of its seeds, which is one of the most important food grade natural colourant widely used in dairy industry, confectionary and bakery products. Apart from this, annatto dye is also used for various non-edible purposes such as cosmeceutical and dying leather etc. From ethanobotanical point of view all parts of this plant is having one or the other use as evident from traditional medicinal practices at various parts of world. Though the economical importance of this plant is its annatto dye, value addition through utility of the plant for various edible and non-edible purposes needs to be documented for commercial applications through farm-based and industrial point of view.

Key Words*:* Annatto dye, Antioxidant, *Bixa orellana*, Bixin, Ethanobotanical, Pigment

INTRODUCTION
Natural colours from plant sources for edible purposes are having great significance from health point of view and also from consumer's acceptance and preference. In view of increasing awareness about various consequences of synthetic dyes, there is a great demand for natural colours. Since many centuries people are using various plant based colours for both edible and non-edible purposes.

Yeats wrote "Good arnotto is the colour of fire" (Natural History, 1870). The red pulp that encompasses the *Bixa* seeds comprises of reddish-orange annatto pigment that has a peppery flavour with a glimmer of nutmeg. Popular in Latino and Caribbean food for flavouring, annatto also finds a notable place in cosmetic industries especially lipsticks thereby gaining the nickname of 'lipstick tree". Red Indians usage of annatto dye to colour skin and traditional dresses has been documented.

Bixa plant is a fast-growing shrub to tree (2-10 m height), native to Neotropics, but widely distributed throughout the tropics (Wealth of India, 1990; Srivastava *et al.*, 1999). The tree produces large clusters of brown or crimson capsular fruit-containing seeds coated with a thin, highly coloured resinous coating that serves as the raw material for the preparation of the annatto colourant.

Earlier review articles on *Bixa* have provided thorough information on the chemistry (Preston and Rickard 1980); extraction methods and formulations (Aparnathi and Sharma 1991); pharmacology and uses (Srivastava *et al.*, 1999); toxicology and processing of annatto (Satyanarayana *et al.*, 2003) and analytical methods to analyze annatto colour (Scotter, 2009). The present review investigates and compiles information in the literature that reveals its potential ethanobotanical application in various parts of globe along with recent findings about its toxicological and safety aspects along with biotechnological intervention for its improvement.

Annatto origin and distribution
Although, native to Tropical America; *Bixa orellana* plants can be found in many regions of world. The genus name, *Bixa*, comes from another Carib plant name usually transcribed as *bija* or *biché*. The species name of this plant is named after the Spanish scientist conquistador, Franscisco de orellana, who is credited with discovering the Amazon River in the 16^{th} century. *Bixa* is also known by other names that are derived from Indio tongues in Central and South America: "*urucul"* from Tupi-Guarani in the

Amazon region, *annatto* from the Carib and *achiote* from Náhuatl in México. *Bixa* belongs to the native flora of Brazil, Paraguay, Colombia, Dominican Republic, Haiti, Trinidad, Mexico, Panama and Hawaii (Ramalho *et al.,* 1987).

It has been traced back to the ancient Mayan Indians, who employed it as a principal colouring agent in foods, for body paints, and as a colouring for arts, crafts, and murals. In 15th and 16th centuries annatto extract was used as body paint by Aztec tribes to rid them from evil spirits and also as an insect repellent. The extracts were later used in Jamaica. Nowadays it is produced in several tropical countries such as Sri Lanka, Indonesia, India and East Africa (Bole, 1995). As an ornamental plant it is cultivated in different parts of India. But organized cultivation is in practice mainly in parts of Orissa, Andhra Pradesh, and Maharashtra and to some extent in Kerala, Karnataka and Tamil Nadu for obtaining annatto dye (Patnaik, 1971).

Botanical description

In Cronquist system, the family *Bixa*ceae includes 3 genera and a total of 25 species. The genus *Bixa* consists of one or four species, depending on taxonomic interpretation (Heywood, 1993). According to Zipcode Zoo (1992) there are 16 species in the genus *Bixa*. On the basis of flower, fruit colour and shape *Bixa orellana* has three varieties: one with white flowers and green capsules, second with purple flowers and brownish-red capsules and third with pink flowers and red capsules (Akshatha *et al.,* 2011). Though some other minor variations in fruit shape or colour reported there is no authentic information available in this regard. Though earlier reports indicates, that the pigment content varies between 5% in hemispheric fruits, 3-3.5% in conical variety and 1.5-2% in ovate variety (ITC, 1990) another study showed more pigment levels in ovate fruit variety (Akshatha *et al.,* 2011). The species has one of the lowest DNA amount (4C = 0. 78 pg) estimates for angiosperms and has 2n = 14 chromosomes (Hanson *et al.,* 2001; Ohri *et al.,* 2004).

B. orellana L. is a bushy shrub with a height ranging from 3 to 10 meters. 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. Leaves are ovate with a round, heart-shaped base and a pointed tip. Petioles were swollen at base and apex. *Bixa* leaves are arranged in a spiral. Leaves on white flower-producing plants were dark green, while those on pink or purple flower-producing were light-greenish in colour. Leaves of pink-flowered plant show dark-red venation contrary to pale-green in purple and white-flowered plants. Leaf blade is waxy in pink-flowered plants, while rough in white-flowered plant (Akshatha *et al.,* 2011). The flowers are white, pink, or purple coloured. *B. orellana* has a panicle type inflorescence with bi-sexual, regular, and hypogynous flowers. Irrespective of their colour all the flowers are five-petalled with numerous stamens developing centripetally. Petals are obovate, distinct, imbricate and dotted (the dots representing the dye cells). The filaments are long, extremely thin, and white or pink in colour with basifixed, dithecous, and horseshoe-shaped anthers corresponding to the petal colour. The gynoecium is bicarpellary and syncarpous. The ovary is unilocular with two parietal placentae bearing numerous ovules (Chopra and Kaur 1965). Qualitative observations during anthesis and stigma receptivity suggested that annatto is protandrous, that is, the anthers shed their pollen before the stigma of the same flower was receptive (Rivera-Madrid *et al.,* 2006). Also, flower maturation was asynchronous on the same panicle and between different panicles in the studied annatto varieties, which agrees with reports by Vallejo (1991). From the flower protrudes a striking two-valved fruit, covered either with dense soft bristles or a smooth surface. These round fruits, approximately 4 cm wide, appear in a variety of colours: green, brownish-green, maroon, and most commonly bright red; and shapes: hemispherical, conical and ovate (Akshatha *et al.,* 2011). When ripe, the pod splits in half to reveal about fifty seeds encased in a red pulp (Howard, 1989; Liogier, 1995).

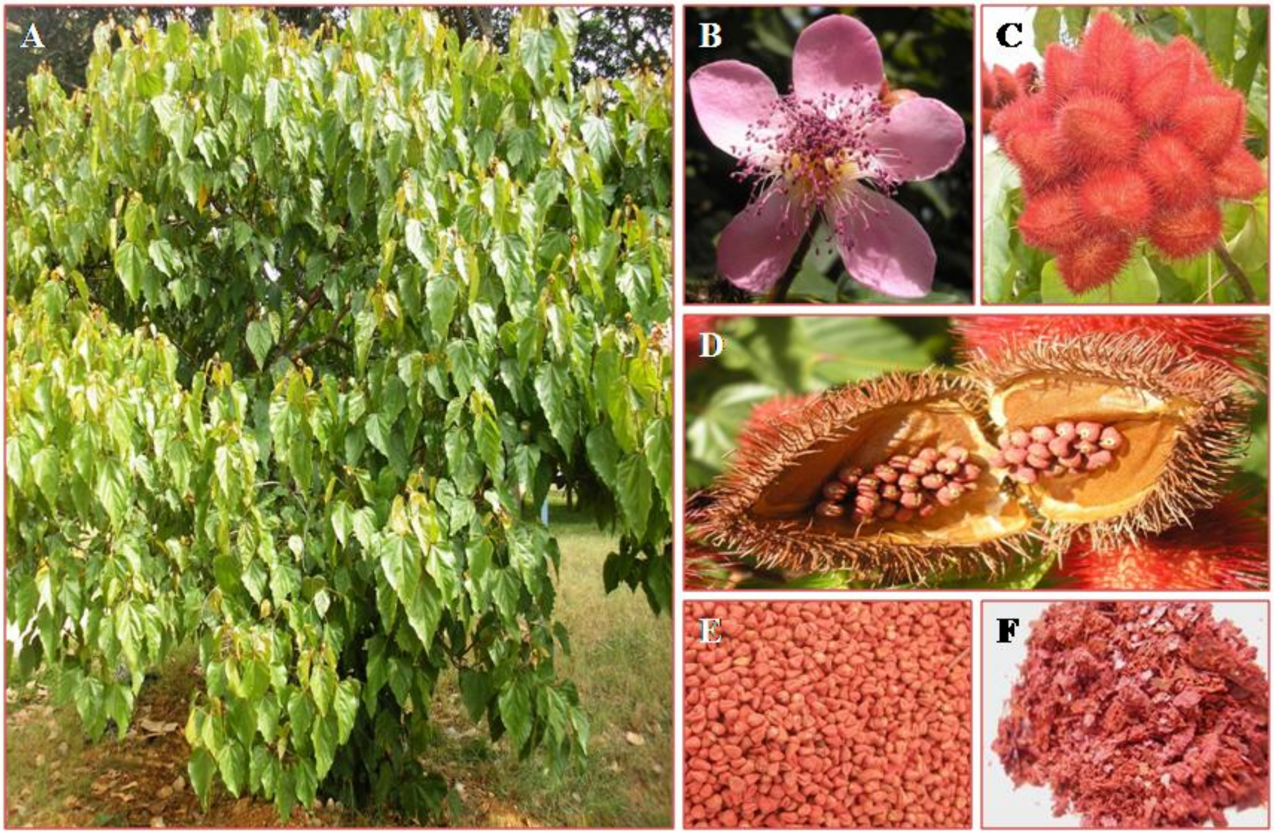

Figure 1: Annatto yielding *Bixa orellana* plant. **A:** Whole plant; **B:** Flower; **C:** Fruit bunch; **D:** Dehiscenced fruit; **E:** *Bixa* seeds and **F:** *Bixa* powder.

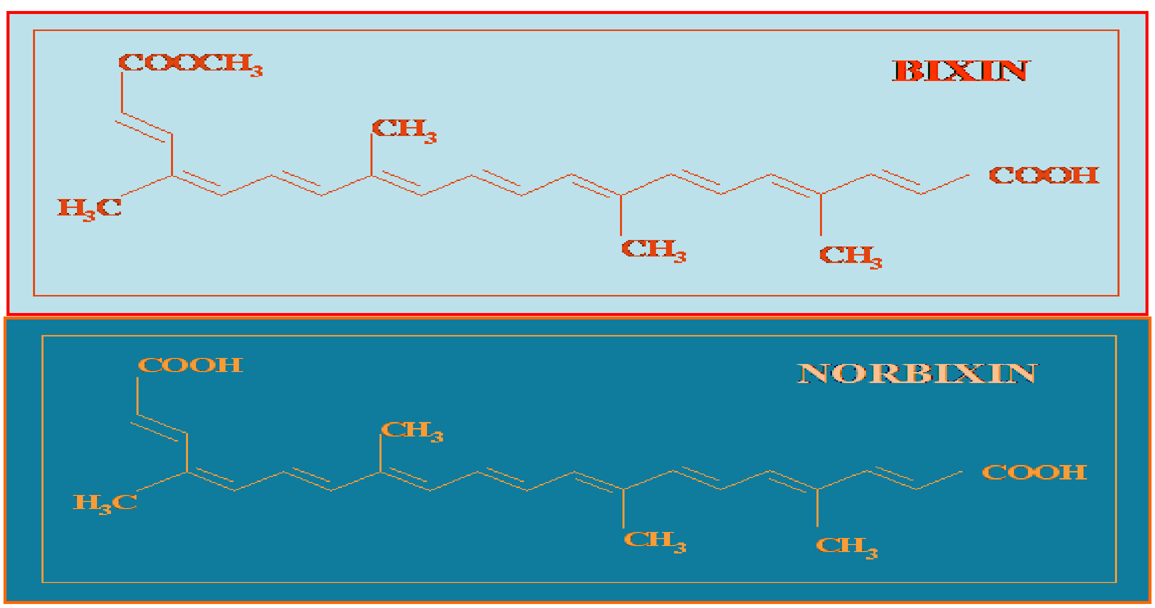

Figure 2: Chemical structure of Bixin and Norbixin.

Agrotechnology for annatto

Bixa orellana is commercially grown for the dye product and for its seeds as a spice. It requires a tropical setting in a loamy soil at altitudes below 1,000 m (3,000 ft). Annatto is considered shade sensitive (Rosalen *et al.,* 1991), and the highest yields are obtained with full sun exposure. Annatto is grown in well drained red and alluvial soils with pH 6 - 7.5. Being a tropical plant it thrives well under 28° to 44° C temperature and 800-1500 mm annual rain fall. It cannot withstand severe cold climates. The economic life of plant is about 20 to 25 years. Land is ploughed, levelled and prepared before planting. Annatto grows best on nutrient-rich clay soils with a pH of 5.5±6 (Bole, 1995; Rosalen *et al.,* 1991). In eastern, Amazonia, annatto was successfully grown on poor soil after many slash-and-burn cycles. Basic information on seed yields, biomass, nutrient accumulation and mineral nutrition of annatto trees growing under agro-forestry conditions with different inputs of fertilizer and lime on a central Amazonian Ferralsol was documented (Elias *et al.,* 2002). Under agro-forestry conditions on a ferralitic Amazonian upland soil, annatto showed a pronounced growth and yield response to increased fertilizer inputs which seemed to be due mainly to improved availability of phosphorus. The planting material can be obtained through seeds, cuttings and tissue culture plants from certified and quality source. The germination percent of seeds is reported to be low (25-30%) due to high percentage of non-viable seeds and mucilaginous secretion. Therefore, cuttings, clonal propagation and tissue cultured plants are preferred. It requires a specific soil rich in Mn and extremely favorable climatic conditions for growth (Sharon and D'Souza 2000). Cuttings taken from flowering plants will produce flowers and fruits and a smaller shrub than plants of seed origin (Bailey, 1941). Earlier report indicates that seeds dried to moisture contents of 10 to 15 percent germinated at from 8 to 58 percent compared to fresh seeds (65 percent moisture) that gave 96 percent germination (Belfort *et al.,* 1992). Scarification was reported to improve germination of fresh seeds in another study (Amaral *et al.,* 1995). No reliable statistics on yield are available. Though the yield of seeds is projected by various reports at 300-600 kg/ hectare it varies with the plant age and climatic and agronomic conditions (ITC, 1993).

Generally flowering starts during the 3rd year of planting. However, tissue cultured plants are reported to be under flowering in 2nd year itself (NABARD, 2007). Blooming starts from July-August to end of October. The fruiting therefore may be seen from September-October to February (NABARD, 2007). Capsules are harvested in bunch. Screening of harvested capsules to discard fungal infested seeds is vital as it will not only rot the seeds but also bring down the quality of seeds. Pruning is important to get better yield from Annatto plant. Details of irrigation, fertilizer's usage, pruning and other agronomic practices for good yield of seeds were reported (NABARD, 2007; TEMEIS Project, 2009). The right stage of harvesting is determined by stage of drying of capsules and development of cracks thereon. To remove moisture if any bunches are spread onto polythene sheets or clothes under semi shade conditions for 6-7 days. The dried pods are beaten to remove the seeds. The separated seeds are winnowed and stored in gunny bags in cool and dry places.

Various bioactives from annatto plant

Bixa plants are commercially grown for the high apocarotenoid pigment content 'annatto' in its seed coat. Annatto, a mixture of eight colourants of carotenoid group can act as a pigment in a number of different chemical forms; the predominant form being bixin (>80% of the total carotenoid content) and also comprises of norbixin, β-carotene, cryptoxanthin, lutein, zeaxanthin and methylbixin (Tirimanna, 1981; Gulrajini *et al.,* 1999; Hallagan *et al.,* 1995; Sekar, 2004; Smith, 2006). In addition to bixin, numerous other pigments which are in minor proportions have been characterized from annatto extracts (Mercadante *et al.,* 1999) including: six apocarotenoids (C30 and C32), eight diapocarotenoids (C19, C22, C24, and C25) and a carotenoid derivative (C14). In fact, *B. orellana* is the richest known source of terpene alcohol, *all*-E-geranylgeraniol which is present to an extent of 57%, or *ca* 1% of dry seeds (Jondiko and Pattenden 1989) and also other isoprenoids such as farnesylacetone, geranylgeranyl octadecanoate, geranylgeranyl formate, and the δ-tocotrienol including flavonoids (including luteolin and apigenin)

(Jondiko and Pattenden 1989; Frega *et al.*, 1998; Pino and Correa 2003). However, the main isoprenoid produced in *B. orellana* is the apocarotenoid bixin (Jondiko and Pattenden 1989; Lancaster and Lawrence 1996; Mercadante *et al.*, 1996).

Phytochemical analysis of *B. orellana* leaves showed the presence of flavonoid bisulfates (Harborne, 1975), sterols, tannins, saponins (Shilpi *et al.*, 2006) and of an essential oil comprising mainly sesquiterpenes with ishwarane as the major compound (Lawrence and Hogg 1973). The roots have been found to contain the triterpene tomentosic acid (Schneider *et al.*, 1965). *Bixa*ghanene, bixein, bixol, crocetin, ellagic acid, isobixin, phenylalanine, salicylic acid, threonine, tomentosic acid, and tryptophan are the other phytochemicals present.

Applications of annatto

Bixin has been used by humans in such a wide range of products because it easily mixes and dissolves into both water-based and oil-based products destined for human consumption and human adornment. From Brazilian herbal medicine to jellies and soft drinks, the list of human uses for this yellow-orange pigment derived from the brilliantly red seed covers of *B. orellana* is long and varied. These pigments are widely used for industrial food and beverages, cosmetics, and as natural dyes for textiles (Chattopadhyay *et al.*, 2008).

World production, economics and market of annatto

Last two decades have seen an increase in demand for annatto and other natural colourants because of the consumer ban on the chemically synthesized azo dye (Collins, 1992). Annatto ranks second in economic importance – behind caramel – with an estimated world consumption of 14,500 tons of annatto seeds with an average cost of US$ 1100/mton of seeds (Satyanarayana *et al.*, 2003).

The main commercial producers of Annatto are countries in South America (Brazil, Peru, Mexico, Ecuador, Dominic Republic), Central America (Honduras, Colombia, Bolivia, Guatemala and Costa Rica), the Carribean (Jamaica, Tobago and Trinidad), Africa (Kenya, South Africa, Zimbabwe and Ivory Coast), and also India and Sri Lanka. World production, estimated at about 3,000 t of annatto seed in 1983 (Anand, 1983), has been rapidly enhanced to 14,500 metric tons (dry weight) per year (Levy and Rivandeneira 2000). Out of this 60.2% from Latin America, 27.4% from Africa and 12.4% from Asia. The major producing countries of annatto are all from South America; Peru is by far the world leader, with Guatemala and Ecuador trailing. Of the African countries, Kenya is the largest exporter, with the Ivory Coast also providing exports. India and Spain are emerging as important exporters to the European market. The United States is an exporter of processed annatto (TJP Market Development 1997).

Brazil is the largest producer with about 5000t. Peru is the largest exporter of annatto seed, annually about 4000 t particularly to Japan. Kenya exports annually about 1500t annatto seeds and extracts. Production statistics are not usually available, and would not provide a reliable guide to international trade since many of the producing countries utilize significant quantities domestically (e.g., Brazil is a large producer and consumer, needing additional imports). The USA is by far the largest importer of annatto products, followed by Europe in which Britain is the biggest importer and Denmark is the biggest processor (Foodnet, 2002).

The bulk of the United States' imports which is about 40 percent of the world's total imports are in the form of *Bixa* seeds (91-93 percent) i.e., 2,500-3,000 metric tons (5.5-6.6 million lbs) with a market value of $2.5-3.3 million, and the rest is in extracts. Jamaica, with the reputation of producing the very best annatto, for reasons possibly to do with labour costs has reduced production over the last decade.

Annual world production of dried annatto seed at the beginning of 21st century is estimated at about 10,000t of which 7000t enters international trade. The average market price per tonne seed varies strongly; between 1984 and 1990 it fluctuated between US$ 600 and US$ 2300. The main market for annatto is the United States with 3000t/year., followed by Western Europe (2500t) and Japan (1500t). Some 70% of the product is used in importing countries to colour cheese. Trade in annatto extracts (instead of dried seeds) has increased strongly since the 1980s, with the water soluble norbixin extract being largest in volume, followed by vegetable oil extracts, and solvent-extracted bixin in third place. Between 1988 and 1992 the import of annatto extracts increased four times more than annatto seed imports (Iqbal, 1993). In Brazilian market during the year 1988, the prices for annatto seeds were 1,500 $ per ton, 30,000 $ per ton for annatto extract with 30% bixin content (FAO, 1995). According to one report (ITC, 2003), the annual production of annatto globally is 10,000 to 11,000 tons and the price of Peruvian annatto seeds was US$700 per ton which is considered as superior quality. Average price of one metric ton of seeds comes to 1,100 $; 1% bixin powder costs around 1,000 $ per metric ton whereas, pure bixin crystals costs 1,07,000 $ per metric ton (Levy and Rivandeneira 2000). Approximately, one metric ton of seeds yield 20 kg of bixin (Satyanarayana *et al*., 2003). Because part of the annatto is processed into liquids of various strength before export, estimating the size of the annatto market is difficult.

The seed and processed annatto products are sold on the basis of the content of the essential chemical ingredients, bixin or norbixin, in the seed. Jamaican seed containing about 3 per cent bixin, for instance, is about twice as valuable as some Indian seed with less than 2 per cent. Caribbean seed also has a greater red, rather than yellow, component in the dye, which adds to the value. According to the FDA, annual consumption of annatto seed as a direct food additive is 3.65 million lbs with an additional consumption of 1.5 million lbs of annatto extract (FDA, 1994).

Annatto pigment for human consumption

As food grade colourant

Among the naturally occurring colourants, annatto ranks second in economic importance (Ghiraldini, 1989). Annatto extracts are generally formulated to impart colour shades in the range red, orange and yellow in different foods (Scotter *et al.,* 1998). Annatto seeds and extracts have been used for over 200 years in Europe and North America to impart a yellow to red colour to foods, especially dairy products such as cheese and butter (Collins, 1992; Evans, 2000) and is considered safe for human consumption, since it has been used as a food colourant by peoples in Latin America for many centuries.

The lower cost of production and the low toxicity, make annatto a very attractive and convenient pigment for the food industry in substitution to the many synthetic colourants that can be mutagenic or carcinogenic (Hallagan *et al.,* 1995; Sasaki *et al.,* 2002). Since the 1800's annatto colour has been used to create the rich golden-orange colour of cheese. Annatto is available commercially in oil-soluble and water-soluble forms, depending on the method of extraction and subsequent preparation into dilutions, suspensions, mixtures, emulsions and powders. It is usually purchased on the basis of bixin content.

Annatto oil also called as Roucou contains antioxidants that are healthy for humans. The light flavour and the great colour that annatto oil adds to all types of food has earned it the nickname "poor man's saffron" since it can give food the same distinctive colouring at a much lower price. Some of the recipes that could be found online using annatto are Cochinita Plib, pork dish Maya style, Achiote Recado, and Maya chicken with orange spicy orange paste.

Oil-based annatto preparations are commonly used to colour foods with high fat content, such as processed cheese or margarine/shortening (Gulrajini and Gupta 1992). They are also used extensively in bakery products, biscuit fillings, popcorn and snack foods, sauces, dressings and cream desserts (Coulson, 1980). Emulsified annatto colours are used in such products as processed cheese, ice cream, soup, confectionery and dairy products (Paumgartten *et al.,* 2002). The more acid stable emulsified annatto finds its use in such applications as juices, liqueurs, transparent jellies and gelatinous desserts. Water-soluble annatto has traditionally been used for colouring of cheese, but is now used in many other applications like puddings, tomato sauce, breakfast cereals, butter milk desserts, chocolate fillings, smoked fish and pet food (Smith, 2006). Annatto (*Bixa orellana* L.) powder is reported to partially replace nitrites in sausages without affecting the microbial or sensory profiles of the finished product (Zarringhalami *et al.,* 2008).

As condiment

Annatto is widely used in the Caribbean and Latin America, especially Guatemala and Mexico. The seeds are also particularly associated with Filipino cuisine, in dishes like; *ukoy*, shrimp and sweet potato fritters; *pipian*, chicken and pork in an annatto oil sauce; and *kari-kari*, a brightly coloured vegetable and oxtail stew (Magness *et al.,* 1971). In the Caribbean, the seeds are usually fried in (animal or vegetable) fat; after discarding the seeds, the then golden-yellow fat is used to fry vegetables or meat. The original Aztec drinking chocolate is reported to have contained annatto seeds as well. In South México (Yucatán), meat is often marinated with a spice mixture called *recado* that derives its vibrantly yellow colour from liberal addition of annatto. Similarly seed powder was used by Philipines in preparing soups and stews and in Vietnam batters and coconut based curries are often prepared with annatto oil to achieve a more attractive colour.

Annatto seeds have been used as a spice in food items like beef, chicken, eggs, fish, legumes, squash, sweet potatoes, sweet peppers, tomatoes, pumpkin, soups, stews, okra beans and rice. Because of its many uses with Mexican dishes and its natural healthy non-fat properties, it has become a favorite of the naturalist and the vegetarians.

Safety assessment

The toxicology of annatto colour was reviewed by Kroes and Verger (2004). Annatto extract does not exert any genotoxicity, subacute and chronic toxicity, reproductive toxicity or carcinogenicity (Zbinden and Studer 1958; van Esch *et al.,* 1959; Haveland-Smith 1981; JECFA, 1982; Hallagan *et al.,* 1995; Fernandes *et al.,* 2002; Paumgartten *et al.,* 2002). The acceptable daily intake (ADI) estimated from a long-term study in the rat, however, is quite low at 0–0.065 mg/kg/day, because of the low concentration of carotenoid in the annatto extract used in previous studies (JECFA, 1982). Annatto pigments are absorbed and rapidly eliminated from the blood in the rat (JECFA, 1982; Levy *et al.,* 1997). Levy *et al.,* (1997) also reported that norbixin (hydrolyzed derivative of bixin) could no longer be detected in plasma 24 hours after ingestion of bixin in human volunteers.

In 2002, the Joint FAO/WHO Expert Committee on Food Additives requested information relating to the toxicity, intake and specifications of annatto (JECFA, 2002). European annatto producers consulted with the food industry to determine use levels of specific annatto extracts. These data were combined with the levels of bixin/norbixin in particular extracts to estimate the concentration of bixin/norbixin in foods. Concentrations in food were combined with data about food consumption using various methods to estimate consumer intakes, which ranged from less than 1–163% of the acceptable daily intake (Tennant and O'Callaghan 2005).

Food formulations using annatto dye

Annatto is one of the oldest safe dyes known to humans and is extensively used by food industries as a natural food grade colourant. Formulations of annatto extract are made to impart orange-yellow colour shades to processed foods (Scotter *et al.,* 1998). Application of water soluble annatto dye and their stability during extrusion cooking has been studied in rice flour (Maga and Kim 1990) and in corn starch (Berset and Marty 1986). Krishnamurthy and Giridhar (1976) used the water soluble annatto dye in orange squash, synthetic syrup, mango pulp and tomato ketchup, and found that the colour is stable for a period of 2 months. Water soluble annatto dye formulation can readily be used for acidic beverages to impart orange-yellow colour at low (Prabhakara Rao *et al.,* 2002). Spray-dried water-soluble and acid-stable annatto dye formulations are also used to colour the fruit and vegetable products such as lime squash, orange squash, mixed fruit jam and tooty-fruity (Satyanarayana *et al.,* 2006). Water soluble annatto dye formulations in the range 15-50mg of norbixin/kg of product are used to colour extruded products like noodles and fryums as well as bakery products like sugar cones (Prabhakara Rao *et al.,* 2007). Similarly the influence of source and quality on the colour characteristics of annatto dye formulations was studied (Satyanarayana *et al.,* 2010). Formulation of annatto feed concentrate for layers, a poultry feed is also reported (Ofosu *et al.,* 2010). Application of annatto formulation to poultry feed enhances the yolk colour of commercial table eggs. The stability of inclusion complexes of bixin for industrial application has been evaluated and it showed that bixin-β-cyclodextrin have a greater ability to provide colour than the pure colourants (Marcolino *et al.*, 2011).

The use of natural pigments requires a chemical knowledge of their molecules and stability in order to adapt them to the conditions of use during processing, packaging and distribution. Microencapsulation by spray drying is the most common method of encapsulation of food ingredients because of it being more economical and of extensive use in the food industry. Microencapsulation by spray drying in different solvents, using the biopolymer chitosan as the encapsulating agent has been reported (Parize *et al.*, 2008).

Stability of the dye during food formulations

Studies were carried out to determine the stability of bixin (oil-soluble dye) during different treatments and processing in traditional foods of India (Prabhakara Rao *et al.*, 2005). The mass fractions of bixin lost were extreme when the dye was exposed directly to heating in a baking oven and in deep fat heating. Stability of natural colourants during extrusion cooking was studied in rice flour by Maga and Kim (1990) which shows that the dyes underwent some loss during extrusion cooking at higher temperature, and the water-soluble annatto dye was found to be more stable. The colour stability of commercial water-soluble annatto solutions was studied by exposing it to heating temperature (Ferreira *et al.*, 1999) and the storage stability of water-soluble annatto formulations in the orange RTS model systems was studied (Prabhakara Rao *et al.*, 2002). Effects of cooking temperature, cooling rates, pH, types of emulsifying salts and the age of cheese on the development of pink discolouration in annatto were discussed (Shumaker and Wendorff , 1998). They noted that higher temperatures caused decreased hunter values and hue angles. Emulsifying salts like sodium citrate also decreased hue values. Addition of whey resulted in pinker discolouration, and it increased with addition of acid.

Illegal usage of annatto dye

The illegal use of annatto to colour milk goes back as far as the early 20th century where it was reported by UK Public Analyst laboratories (Ellis Richards, 1923; Collingwood Williams, 1925). Annatto is currently permitted in the EU for the colouring of certain margarines and cheeses but is not permitted for the colouring of milk cream or butter (EC 1994 as amended). Annatto/bixin/norbixin is permitted colours under the European directive 94/36/EC on colours for use in foodstuffs. Annatto is permitted for use in food commodities such as savoury snack products, coated nuts, extruded products and flavoured breakfast cereals, but it is not permitted for use in spices. However, amongst other non-permitted dyes bixin was detected in 18 of 893 samples of spices, sauces and oils by UK enforcement laboratories during 2005-2006 as part of the UK Imported Food Programme (Food Standards Agency, 2006). An alert on the annatto dye recall was made to Europe's RASFF (Rapid Alert System for Food and Feed) because of the unauthorised presence of colour annatto/bixin/norbixin in palm oil, sweet chilli and chilli which created confusion in the food industry. European Commission clarified this by providing a list of food stuffs that could be coloured using annatto, bixin and norbixin.

Adulteration of annatto dye

Annatto, a colouring matter obtained from *Bixa* is used to colour milk and other food products, soaps, cosmetics, textiles, leathers and paints. It is known to be adultered with various organic and inorganic substances (www.foodadulterationinfo.com/.../how-colouring-agent-annatto-being-adulterated). The common organic annatto adulterants are barley, rye, turmeric and wheat flours. The inorganic adulterants are sulphate of lime, alkali, salt, carbonate of lime, an oily substance probably soap, red ferruginous earths, mostly venetian red, copper and red lead. Since lime and flour are known to reduce the colour of annatto; salt, alkalies and red earths are added to imitate the genuine annatto. Organic adulterants of annatto are detected by microscope and appearance, weight and taste of the ash is used to detect inorganic adulterants.

Annatto is commonly used to colour cheese and its adulteration in cheese can be detected by comparing the colour of cheese with uncoloured ones (www.foodadulterationinfo.com/.../how-colouring-matter-used-adulteration-cheese-can-be-detected). The adulterants are also identified microscopically and by chemical analysis. Annatto is also adulterated with ochre, sand gypsum, and a farinaceous matter (Greive, 1995).

Ethanobotanical/Medicinal uses

In traditional practices

Annatto extracts from leaves, roots and seeds have traditionally been used for medicinal purposes by South American Indians for the treatment of wounds and to treat diarrhoea and asthma (Lauro and Francis 2000). The pulp surrounding the seed 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). In Brazil annatto extract is used for heartburn and as a mild diuretic. The entire plant is used against fever and dysentery. A decoction of the leaves is used to stop vomiting and nausea; treat heartburn, prostate and urinary difficulties, and stomach problems (www.tropilab.com/*Bixa*-ore.html).

A tea made with the young shoots is used by the Piura tribe of Rainforests of Amazon as an aphrodisiac and astringent and to treat skin problems, fevers, dysentery, and hepatitis (Terashima *et al.,* 1991). The leaves are used to treat skin problems, liver disease, and hepatitis. The Cojedes tribe uses an infusion of the flowers to stimulate the bowels and aid in elimination as well as to avoid phlegm in newborn babies. Traditional healers in Colombia have also used annatto as an antivenin for snakebites. The seeds are believed to be an expectorant, while the roots are thought to be a digestive aid and cough suppressant (Dunham and Allard 1960). Its efficiency against STD such as gonorrhea was reported (Caceres *et al.*, 1995).

Leaf decoctions are used to treat heartburn and stomach distress, as a mild diuretic and mild laxative and to treat burns in Brazilian herbal medicine and also in Peruvian herbal medicine to treat prostate disorders and internal inflammation, arterial hypertension, high cholesterol, cystitis, obesity, renal insufficiency, and to eliminate uric acid.

Hepatoprotection

Methanol extract of *B. orellana* seeds illustrated hepatoprotective activity against Swiss albino rats with liver damage induced by carbon tetrachloride (CCl_4) (Ahsan *et al.,* 2009). Blood samples of carbon tetrachloride treated rats showed significant increase in the levels of serum enzyme activities, reflecting the liver injury caused by CCl_4 and blood samples from the animals treated with the methanol extracts of *B. orellana*, showed significant decrease in the levels of serum markers, indicating the protection of hepatic cells.

Diabetes mellitus

Although *Bixa orellana* has been used for the treatment of diabetes mellitus; preliminary studies have shown that a crude extract of annatto seed exhibited either glucose lowering or hyperglycaemia-inducing activity depending on how it was further manipulated (Morrison *et al.,* 1991). The effects of this glucose-lowering extract on C-peptide and streptozotocin-induced diabetic dogs has been investigated and it was found to decrease blood glucose levels in fasting normoglycaemic and streptozotocin-induced diabetic dogs (Russel *et al.,* 2008). *B. orellana* lowered blood glucose by stimulating peripheral utilization of glucose, and it is possible that this glucose-lowering extract might be of pharmacological importance (Russel *et al.*, 2008).

Antimicrobial activity

Huhtanen (1980) and Galindo-Cuspinera *et al.,* (2003) reported that annatto extract exhibits antimicrobial activity against strains of *Clostridium perferingens* and *C. botulinum*. The extracts of the leaves and seeds of *Bixa orellana* showed a broad spectrum of antimicrobial activity (Irobi *et al.,* 1996; Castello *et al.*, 2002; Fleischer *et al.,* 2003) thereby providing scientific support for the use of *B. orellana* in traditional medicine as a gargle for sore throats and oral hygiene.

Bixa orellana leaves exhibits antifungal activity (Freixa *et al.*, 1998) and barks exhibit antigonorrheal activity (Caceres *et al.,* 1995). Similarly extracts of flower and leaf extracts demonstrated *in vitro* antibacterial activity against several bacteria, including *E. coli*, *Shigella dysenteriae* and *Staphylococcus* causative agents of diarrhoea and dysentery (Shilpi *et al.*, 2006).

Antioxidant activity

Among the natural carotenoids, bixin is one of the more effective biological singlet molecular-oxygen quenchers and may contribute to the protection of cells and tissues against deleterious effects of free radicals (Di Mascio *et al.,* 1990). Bixin is also an effective inhibitor of lipid peroxidation (Zhang *et al*., 1991), inhibited TBARS production in rat peripheral macrophages, and this could be the mechanism by which carotenoids *in vivo* protect cells and tissues from damage induced by oxygen metabolites (Zhao *et al.,* 1998). It is an antioxidant inhibitor of lipoxygenase activity (Canfield and Valenzuela 1993). Methylbixin has shown enhancement activity of gap junctional communication which is important in cancer prevention (Zhang *et al*., 1991).

Effects of annatto as an antioxidant were also investigated by Haila *et al.,* (1996) and their studies on the formation of hydroperoxide of triglycerides in the presence of γ-tocopherol and annatto extracts revealed that both the extracts inhibited the formation of hydroperoxide. Addition of γ-tocopherol retarded the loss of carotenoid, and hence this combination was more effective in inhibiting the hydroperoxide formation. This particular principle is having significance in making food formulations with annatto colour, wherein it enhances pigment stability.

Protective effect

In vivo studies have provided evidence of the bixin modifying potential against chromosomal damage induced by radiation in mouse bone marrow cells (Thresiamma *et al.*, 1998), by cisplatin in rats (Silva *et al.*, 2001), radioprotective effect in bone marrow cells of gamma irradiated mice (Karchuli *et al.*, 2009) and clastogenic effects of antitumor agents (Antunes *et al.,* 2005) suggesting it as a promising agent against radiations.

Similarly norbixin has been shown to have antigenotoxic properties based on its protection of *Escherichia coli* cells against DNA damage inducers, UV radiation, hydrogen peroxide (H_2O_2), and superoxide anions ($O_2^{\cdot -}$) (Kovary *et al.*, 2001), and it also displayed antimutagenic properties (Júnior *et al.*, 2005).

The activity of annatto (*Bixa orellana* L.), a natural food colourant rich in carotenoid, on the formation of aberrant crypt foci (ACF) induced by dimethylhydrazine (DMH) in rat colon and the effect of annatto on DMH-induced DNA damage, by the comet assay has been evaluated (Agner *et al.,* 2005); and these findings suggest that annatto may have chemo-preventive effects.

Other medicinal uses

Methanolic extract of *Bixa* leaves exhibited neuropharmacological, anticonvulsant, analgesic, antidiarrhoeal activity (Shilpi *et al.,* 2006) and diuretic activity (Radhika *et al.,* 2010). Neuropharmacological investigations reveal that the extract statistically reduced the time for the onset of sleep in pentobarbitone-induced hypnosis test and decreased locomotor activity in open-field and hole-cross tests.

The seeds are cordial, astringent, febrifuge and a good remedy for gonorrhea (Yusuf *et al.,* 1994; Kirtikar and Basu 1999). The root bark is also useful in gonorrhea (Yusuf *et al.*, 1994; Ghani, 2003) and as an antiperiodic and antipyretic (Kirtikar and Basu 1999). The whole root extracts have been reported to have spasmolytic activity (Mans *et al.,* 2004). An infusion of the leaves and roots is useful in epilepsy, dysentery, fever and jaundice (Perry, 1980; Joshi, 2000; Ghani, 2003). Previous pharmacological studies have revealed that *B. orellana* extracts possess antiprotozoal, anthelmintic and platelet antiaggregant activity (Villar *et al.,* 1997; Barrio *et al.,* 2004). Extracts of leaves and branches have shown to be effective at neutralising the effects of snake venoms (Nunez *et al.*, 2004).

Uses other than it's consumption

Although annatto has wide applications in food industry as a colour additive it also finds its usage in cosmetics, textiles and pharmaceutical products (Póvoa, 1992). The reddish-yellow pigment coating of annatto seeds is still used by Brazilian indigenous people to paint their bodies, and to dye their breechcloths (Plotkin, 1993).

As dye

Achiote (*Bixa orellana*) has been traditionally used for face and body painting (Paumgartten *et al.*, 2002) mainly by people in Suriname and also by Tsáchila people across the Andes on the western slopes, near the Ecuadorian town of Santo Domingo de los Colourados. Bixin was also the original Amerindian war paint (Mabberly, 1987).

The dye is also used to colour the textiles (Morton, 1981; Morton and Ledin 1952) and fabrics and the weapons with it (Schultes and Raffauf 1990). Dyeing of silk and wool with annatto is found to be effectively accomplished commonly at Ph ~ 4.5. In India also it is popular as 'Dhobi dye' used by some communities (washerman) to mark the clothes while washing. In the Philippines, the red pulp from the seeds is used in the polish for russet leather (Quisumbing, 1951). Annatto has remarkable affinity for both the protein fibres. Pre application of ferrous sulfate on wool and silk followed by dyeing with annatto produces most balanced improvements in colour uptake, light and wash fastness and colour retention on repeated washing of such protein fibres (Das *et al.*, 2007).

As cosmeceutical

Annatto is being used increasingly in body care products. European Union rules concerning cosmetic products allows the use of annatto dye in beauty products (Biezen *et al.*, 2006). Annatto oil is an emollient, and its high carotenoid content provides antioxidant benefits on body care products, while adding a rich, sunny colour to creams, lotions, and shampoos. Similarly annatto paste filters out the ultraviolet rays of sunlight, thereby protecting the skin from excessive sunburn. Dyes for lipstick are also obtained from *Bixa orellana* L. (Siva, 2007) hence, the name lipstick tree. It is also used as a colouring agent for the preparation of sindoor which is an important cosmetic item for married women, worship and other purposes in India (Kapoor *et al.*, 2008). This safe, non-toxic, eco-friendly natural dye-based sindoor is an efficient option for replacing synthetic dye-based sindoors that is known to cause hair loss, edema, erythema and even skin cancer on its prolonged usage (Kapoor *et al.*, 2008).

As pharmaceutical

Annatto is commonly used as a colouring agent for pharmaceutical ointments and plasters (Natural Standard Professional Monograph, 2011). It has been used in direct compression tablet coating and oral liquid drugs (Dinda *et al.* 2008). Complexing bixin with α-cyclodextrin is an efficient way to protect the pigment from environmental and harmful agents (Lyng *et al.* (2005). The complexed form of bixin is more resistant than free bixin to the damage caused by light and air or their combination besides and shows improved water solubility as required for novel formulations of pharmaceutical interest (Lyng *et al.*, 2005).

As fuel

The wood of *B.orellana* is lightweight (specific gravity 0.4), weak, and not durable. It was used in former times to start fires by friction.

Bixa fruit pericarps that are by-product of annatto colour extraction industries can be used as a potential source of fuel (Parimalan *et al.*, 2007a). Approximately of 2.5 kg of dried fruits yield 1 kg of seed which is used for dye extraction thereby leaving 1.5 kg of pericarp unutilized. The estimated yield of annatto fruit is in the range of 800-1500 kg/ ha and the seed yield ranges from 300-600 kg/ha. Therefore, 500-900 kg/ha of pericarp goes to waste. World production of annatto seeds is estimated to be 14 500 t/y on a dry weight basis (Levy and Rivandeneira 2000) which shows that an average 21 400 t of pericarp are disposed off, and the same can be used as a fuel source.

Higher heating values for softwood are 20-22 MJ/kg, and those for hardwood are 19-21 MJ/kg (Demirbas, 1997). Annatto pericarp shows an LCV of 16.224 MJ/kg, which is also equally valuable when compared with the hardwood values. The lower calorific values of annatto pericarp are higher than some plant biomass that is usually used as a source of fuel. Therefore it could be a supplementary source of fuel for third world countries (Parimalan *et al.*, 2007a). Due to its potential as a fuel, it will add value addition to the farmers by fetching good price as an alternate income apart from seeds that are used for extracting annatto dye. Especially under social forestry programs this would be a good choice as it will benefit the planters in various forms.

As a fodder

Bixa meal, which remains after extraction of the pigment from the seed, is a useful additive to poultry feed and can replace 30% of the maize in the food. However, the seed embryo contains a poisonous alkaloid, so it is not wise to use the residues from the extraction process directly (Orwa *et al.*, 2009). Annatto seeds contains about 40-45% cellulose, 3.5-5.5% sucrose, 0.3-0.9% essential oil, 3% fixed oil, 4.5-5.5% pigments (comprised of 70-80% bixin), 13-16% protein, and other constituents. It also contains tannins, ethereal oils, saponins, mustard oil-like substances and mono- and sesquiterpenes (The Raintree Group, 1997; BIBRA, 1986). The high percentage of carbohydrates makes the annatto seed a good alternative as a feed for livestocks. A study was made by Senthil Kumar *et al.* (2007) to estimate nutritional quality and suitability of pigment extracted annatto seed for feeding of ruminant livestock as an alternative to conventional feed ingredients and it was found that pigment extracted annatto seed meal can be used as substitute for cereal grains in the rations of buffaloes as a source of energy and by-pass protein.

Miscellaneous uses

Bixa plants are also grown as an ornamental, particularly the varieties with bright pink pods. Branches with the dry pods are used in dry floral arrangements (Warren, 1997). Bark from the branches of the *Bixa* plant yields a water-soluble gum that is similar to gum Arabic (Orwa *et al.*, 2009). Bixin extracted from the seed coat is used in India as an insect repellent. The seeds are given to bulls to make them aggressive for bullfighters and are taken by Indians as an aphrodisiac (Morton, 1981). A fiber may be obtained from the bark (Quisumbing, 1951). Ropes and twine were made from the fibrous bark (Little *et al.*, 1974).

Improvement of annatto though biotechnological interference

In spite of the production and productivity, annatto pigment content in seeds also accounts for the market value of annatto pigment. Hence, seeds with pigment content more than 2.5% are considered as international benchmark for annatto pigment export (Heywood, 1993). Since, annatto is a perennial cross-pollinated crop (Pavia Neto *et al.*, 2003a), mass production of elite lines and enhancement of annatto pigment is desired. Also, production of annatto seedlings is much delayed with low seed viability (20%) and poor germination (5%) due to dormancy D'souza and Sharon 2001). Hence, *in vitro* regeneration protocol through tissue culture will help in producing the elite clones for planting at commercial scale in a short duration.

Though tissue culture methods can be used to produce elite clones of *Bixa orellana* it has its own limitations. It is a time consuming process although shorter than conventional breeding. To meet the international benchmark of more than 2.5% bixin in seeds, it appears feasible to study and regulate bixin biosynthetic pathway.

Mass multiplication of Annatto plants through *in vitro* technology

The content of bixin generally varies from plant to plant due to its cross-pollinated nature (Aparnathi *et al.*, 1990). In *B. orellana*, due to low seed viability (20%) and poor germination (5%), *in vitro* studies possess commercial value (D'Souza and Sharon 2001). Commercial plantations of this important species are raised through seedlings (Aparnathi *et al.*, 1990). Since the bixin or annatto yield tends to vary from sample to sample, and from plant to plant, high yielding lines should be selected and vegetatively propagated for commercial plantations. Conventional propagation via cuttings has limitations because of the intense leaching of a gummy substance and phenolics from the cut ends, which obscure rooting. Hence, *in vitro* propagation of *B. orellana* could be the answer to get more planting material. *In vitro* regeneration of *Bixa orellana* can also be used for the production of high yielding and productive genotypes thereby accelerating true-breeding programs.

There are several *in vitro* protocols for organogenesis or regeneration of *B.* (Ramamurthy *et al*., 1999; D'Souza and Sharon 2001; Paiva Neto *et al.*, 2003a; Parimalan *et al.*, 2007b, 2008, 2009, 2011a). Studies on the regeneration of plantlets from axillary buds of seedlings (Ramamurthy *et al.*, 1999, D'souza and Sharon 2001) and through intervening seed callus (Sha *et al.*, 2002) have been reported. An efficient micropropagation protocol for annatto (*Bixa orellana* L.) was showed using nodal shoot tip explants by triacontanol (Parimalan *et al.,* 2009). Apart from obtaining high frequency of *in vitro* shoots, TRIA treated plants also showed increase in bixin content in the seeds of annatto under field conditions, which is essential for export. Similarly *in vitro* regeneration of annatto was achieved at a shorter period by an efficient two-stage plant regeneration protocol; where Phenylacetic acid (PAA) and N^6 – benzyladenine (BA) exhibited synergistic effect on shoot multiplication and elongation (Pariamalan *et al.,* 2008).

Direct shoot organogenesis was attained in *Bixa orellana* by inoculating nodal shoot-tip explants on MS medium containing BA and Indole -3- butyric acid (IBA), and supplemented with either putrescine or silver nitrate (Parimalan *et al.*, 2011a). The standardization of somatic embryogenesis helps to maintain and enhance the multiplication of elite clones of interest for higher productivity and economic benefits, and also for the establishment and utility of a transformation protocol for genetic engineering studies to regulate the biosynthetic pathway (Kumar *et al.*, 2006, 2007). Direct somatic embryogenesis of *Bixa* was first established by Paiva Neto *et al.,* (2003b) wherein high frequency of embryogenesis was obtained on medium containing 2,4-D, kinetin, and activated charcoal. Although it showed high frequencies of histo-differentiation at all embryonic stages, the conversion frequency from somatic embryos to normal plants was very low (Paiva Neto *et al.*, 2003b). A more efficient method for direct somatic embryogenesis was established by Parimalan *et al.*, (2011b). Immature zygotic embryonic stalks when inoculated onto MS medium containing B5 vitamins supplemented with BA, NAA, GA3, TIBA and TRIA gave a maximum of 28 somatic embryos in 16 to18 weeks. Callus-mediated methodology was found to be an efficient way to produce greater number of indirect somatic embryos in a short period (Parimalan *et al.*, 2011b). Callus initiation was obtained by inoculating hypocotyl explants on MS medium supplemented with NAA and BA. Indirect somatic embryos were produced within 3 months when callus was inoculated onto MS medium supplemented with BA, AgNO3, and TRIA.

Alternate mode of annatto pigment production

Normal root cultures of *B.orellana* capable of producing annatto has been established under *in vitro* conditions on MS medium containing α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) at 0.05–0.2 mg l^{-1} (Mahendranath *et al.*, 2011). This normal root culture system is an alternative means for annatto pigment production throughout the year and is valuable for the sustainable utilization of this plant for its bioactive ingredients.

Elicitor-mediated enhancement of annatto pigment

Elicitation is a process used to induce the production of secondary metabolites due to the fact that most plant systems produce small amounts of secondary metabolites. Elicitors such as fungal wall materials, plant and microbial polysaccharides, and some chemicals increase secondary metabolite production in various plant cell and tissue culture systems (Johnson *et al.*, 1991). Since annatto plant is of perennial nature, the commercial plantations with pigment yield less than 2.5% will be of low economic value and since replacement of the whole plantation with high pigment yielding varieties will cost much and also maintaining the same plants will fetch less profit due to low economic value. To overcome this problem, elicitation technology will be of very much helpful to overcome this dual problem and thereby maintaining the same plantation to obtain the higher pigment yields. An elicitor mediated approach for enhancing bixin has been reported (Parimalan *et al.*, 2005). It was also shown that using abiotic elicitors as a tool to improve the annatto pigment yield on standing crop (Giridhar and Parimalan 2010) is feasible. Around 3 fold increase of annatto pigment was achieved by using 0.5% extract of *R. oligosporus*. The influence of an algal polysaccharide laminarin on augmentation of annatto pigment in *B. orellana* was studied (Mahendranath *et al.*, 2011). A 2 fold improvement was observed in the pigment content when 1% of laminarin was used as spray.

Regulation of bixin biosynthesis pathway

Recently several methodologies for molecular biology studies of *B. orellana* (Chi-Manzanero *et al.*, 2000; Narváez *et al.*, 2001a) have been developed. Some of the genes involved in isoprenoid biosynthesis have been characterized (Narváez *et al.*, 2001b) for future use as molecular tools in improvement of annatto production. In a study using standard molecular techniques and a bacterial complementation system, the genes involved in the biosynthesis of bixin were cloned and functionally characterized by Bouvier *et al.*, (2003). The pathway determined by the gene isolation has been reviewed in the recent literature (Auldridge *et al.*, 2006). In brief; the lycopene cleavage dioxygenase (*lco*) acts on lycopene to produce bixin aldehyde followed by its conversion to nor-bixin by bixin aldehyde dehydrogenase (*badh*) and finally to bixin by norbixin carboxy methyl transferase (*n-cmt*). Bixin methyl ester accumulated to moderate levels in the heterologous host, demonstrating the function of the genes in the production of the pigment (Bouvier *et al.*, 2003). Bouvier and co-workers propose that these three genes may be sufficient to genetically engineer the accumulation of bixin in tomato, which ordinarily accumulates high levels of lycopene.

The expression of some upstream and downstream carotenoid enzymes in two cultivars of annatto to understand some of the genetic basis governing carotenoid production was investigated (Rodríguez-Ávila *et al.*, 2010). The 3D structure of the enzyme, lycopene cleavage dioxygenase was predicted by MODELLER program and the missing side-chains were verified by SCRWL4 tool (Satpathy *et al.*, 2010). The levels of expression and regulation of bixin biosynthetic genes are still not known. Apocarotenoid pigment bixin is known to be unique to *Bixa orellana*, but according to a recent report they may also be produced by saffron and grapes (Siva *et al.*, 2010). By comparative genome sequence analysis, bixin biosynthetic genes were shown to be present not only in *Bixa* but also in *Crocus* and *Vitis* (Siva *et al.*, 2010). Although presence of bixin were evident in these two plants through TLC, FT-IR and GC-MS studies, further confirmation is warranted through molecular techniques.

Genetic transformation

Genetic improvement of *B. orellana* by conventional breeding is difficult because it is restricted by a long reproductive cycle and the limited genetic variability within the available germplasm (Aparnathi *et al.*, 1990). An alternative to overcome these limitations is the introduction of new traits by *Agrobacterium*-mediated genetic transformation, which is the most commonly, used method for transferring genes into plants cells (Gelvin, 2000). The first report on annatto susceptibility to *A. tumefaciens*-mediated genetic transformation was given by Zaldívar-Cruz1 *et al.*, (2003). Hypocotyls from seedlings of two annatto varieties on inoculation with *Agrobacterium tumefaciens* harboring a binary vector pCAMBIA2301 showed transient *gus* gene expression between 3 and 12 days after inoculation, thereby indicating for the first time that the transfer of T-DNA via *Agrobacterium tumefaciens* is possible in annatto (Zaldívar-Cruzl *et al.*, 2003). An efficient method for *Agrobacterium*-mediated transformation of *B. orellana* was achieved by using somatic embryos derived from direct and callus-mediated methodology (Parimalan *et al.*, 2011b). Somatic embryos treated with *A. tumefaciens* GV 3101 harboring pCAMBIA 1305.2 exhibited a transformation frequency of 2.56% proving its high competency and stability. This somatic embryo-based transformation protocol for the commercially important dye-yielding tropical plant *B. orellana* is useful for its improvement through genetic engineering.

Future perspectives

In view of various edible and non-edible application attributed to this annatto plant establishment of substantial scientific evidences to support its ethanobotanical application is warranted so that in future this plant could be used in making formulation for health food and illness apart from its annatto dye as a natural colourant. In order to identify the elite varieties from different fruiting varieties, studies based on systematic botany, chemotaxonomy and molecular methods are helpful. Particularly the chemotaxonomic markers if any would be a tool for characterization of existing germplasm of *B.orellana*. The various water soluble polysaccharides from the characteristic gum produced from the tender leaved and bark of the stem needs to be studied for identification of germplasm and also value addition. Similarly, there is a great scope to explore and extract bioactive compounds from different parts of the plant i.e., root, stem, bark, leaf, flower, fruit and seed that could be useful as nutraceuticals. Being a commercially important dye yielding plant sustainable production of annatto seed yield is important from economical perspective. In order to improve the yield of pigment molecular method intervention will provide an opportunity not only to screen the germplasm to identify the elite variety through RAPD markers and other marker registered selection but also to regulate the bixin biosynthetic pathway. As heterologous expression of bixin biosynthetic pathway genes demonstrated many folds of bixin production now it could be possible to augment annatto pigment production through genetic engineering. In this regard differential expression of bixin biosynthetic pathway genes during ontogeny of flower and fruit would be helpful to identify the key gene and its subsequent overexpression.

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