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Mentha species: I*n vitro* Regeneration and Genetic Transformation

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Abstract

***Mentha* is a genus of aromatic perennial herbs belonging to the family Lamiaceae. It is distributed mostly in the temperate and sub-temperate regions of the world. Several *Mentha* species are considered industrial crops as they are a source of essential oils enriched in certain monoterpenes, widely used in food, flavour, cosmetic and pharmaceutical industries. *Mentha* has a large number of species that differ widely in their characteristics and polyploidy level. It is known to comprise about forty recognizable species. Utilizing crosses between *Mentha* species, it has been shown that single Mendelian gene(s) control the presence or absence of major compounds such as carvone, menthone, menthol and piperitone. The monogenic basis for conversion of menthone to menthol showed that gene *R*, either homozygous (*RR*) or heterozygous (*Rr*), is responsible for the reduction of menthone to menthol or carvone to carveol. Enzymes involved in monoterpene biosynthesis are described. Pests and pathogens of *Mentha* that cause substantial damage to the crop and considerable loss in oil yield are also described. Termites, cutworms, whitefly and semiloopers damage different parts of the *Mentha* plant. A number of microbes, viz. *Macrophomia phaseoli* and *Puccinia menthae*, also damage the *Mentha* plant. Control measures such as the use of insecticides, disease-inhibiting chemicals, and breeding of pest and pathogen resistant plants are reviewed. *Agrobacterium* genes responsible for the transfer of DNA, and selectable marker genes are discussed. Plant transformation technology has not only played an important role in introducing insecticidal genes into relevant crops but also has become a versatile platform for cultivar improvement as well as for studying gene function in plants.**

Introduction

Several mints of *Mentha* species are industrial crops, a source of essential oils enriched in certain monoterpenes that are widely used in food, flavour, cosmetic and pharmaceutical industries. Different species of mint are used across the globe for their medicinal and culinary properties. Mint is usually taken after a meal for its ability to reduce indigestion and colonic spasms by reducing the gastrocholic reflux. Less well recognized is peppermint's potential role in the management of numerous medical problems, including colonoscopy (Spirling and Daniels, 2001) .The genus *Mentha* has a large number of species that differ widely in their characteristics and ploidy level. Since they are often perennial and produce suckers, *Mentha* species reproduce both by reproductive and vegetative means. In *Mentha* crops, the purity of cultivars is maintained by vegetative means of propagule generation. Mint species are widely distributed and are prone to attack by a variety of diseases and pests. Many laboratories worldwide are carrying out research and development work on mint species towards improvement in the yield and quality of essential oils, and incorporation of disease and pest tolerance and increasing the propagule productivity. Peppermint oil is the major constituent of several over the counter remedies for symptoms of irritable bowel syndrome (IBS). Results of clinical trials indicated that it could be efficacious for relief in IBS (Pittler and Ernst, 1998). In this review, the authors aim to correlate various aspects of *in vitro* regeneration and genetic transformation in *Mentha* species.

Origin and distribution of mint species

Mentha is a genus of aromatic perennial herbs belonging to the family Lamiaceae, distributed mostly in temperate and sub-temperate regions of the world. The number of taxonomically valid species in the genus remains a matter of speculation as members freely cross amongst themselves, producing many intermediary forms. Polyploidy has also played an important role in the process of speciation in this genus. Most of the commercially important mints are hybrids or amphiploids. *M. piperita,* the peppermint, is a sterile first generation hybrid between *M. spicata* and *M. aquatica* (Hefendehl and Murray, 1972). The spearmint, *M. spicata,* is a hybrid of *M. longifolia* and *M. rotundifolia*. Morphological, cytological and biochemical data have shown that the tetraploid species of M*. spicata* (2n=48) originated by chromosomal doubling of hybrids between the two closely related and inter-fertile diploids, *M. longifolia* and *M. suaveolens* (Harley and Brighten, 1977). The bergamot mint, *M. citrata,* is considered to be a variety of *M. aquatica*. It is believed that *M. arvensis* var. piperascens, Japanese mint, is a hybrid between *M. arvensis and M. aquatica*.

Mint cultivation is widely distributed. The United States of America is the main producer of peppermint and spearmint oils. The peppermint (*M. piperita*) is cultivated on a large scale in the states of Oregan, Indiana, Idaho, Ohio and Michigan (Chambers and Hummer, 1992),

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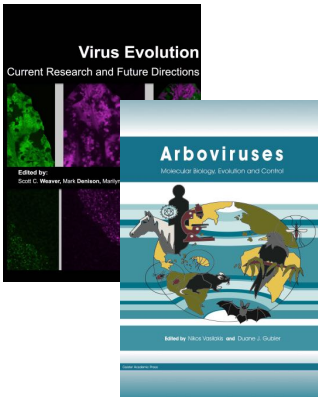

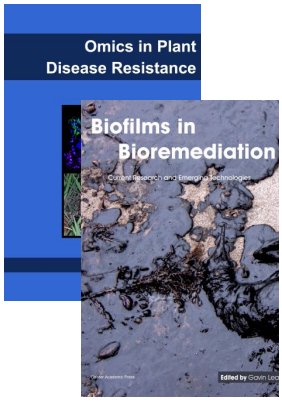

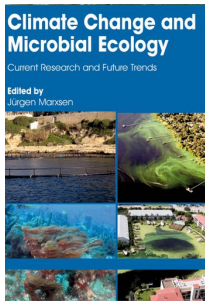

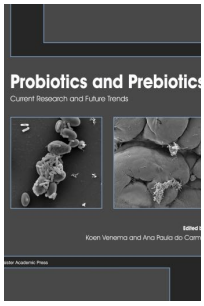

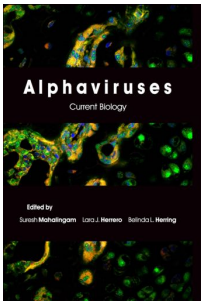

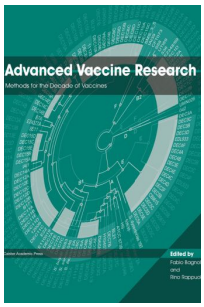

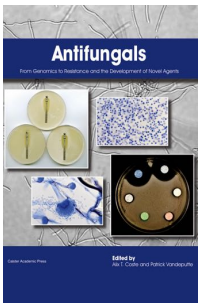

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whereas spearmint cultivation is localized in Indiana and Michigan. Spearmint is also cultivated in France, the United Kingdom, Italy, Yugoslavia, Hungary, Bulgaria, Russia, South Africa, Thailand and Vietnam. Bergamot mint is commercially cultivated in China, Taiwan and India, and menthol or Japanese mint in India, China, Taiwan, Thailand, Japan and Brazil.

Mint essential oil compositions

Mints are cultivated for their essential oils, yielded by distillation of over-ground herbaceous foliage. The oil contains a large variety of aromatic chemicals in varying composition. The oils and their fractions are in great demand world-wide. The constituents of the essential oils of important mint species are listed in Table 1.

Table 1: The uses and essential oil constituents of important mint species

Sl. No.	*Mentha* species	Percent oil in foliage and chemical constituents in oil	Use(s)
1.	*Mentha aquatica*	Oil 0.8%; 40% menthofuron, 28% menthol, 22% menthelacetate, menthone, pulegone, piperitone (in traces)	Flavouring foods and liquors and in medicine for digestive purposes and for gall and stomach disorders
2.	*Mentha arvensis*	1.3-1.6% oil; 70-80% menthol, 10% menthyl acetate, 8% menthone, limonene, pinene, caryophyllene (in traces)	Cold remedies, cough drops, dentrifices, mouthwashes, flavoured tobacco, chewing gum, scented cigarettes and cosmetics. It acts as a carminative, antiseptic and stimulant and is used in preparation of syrups, ointments and balms for rheumatic pain and fevers. The oil has been used in both eastern and western cultures as a folk medicine for treating indigestion, nausea, sore throat, cold, toothache, cramps and ear aches, as an antiseptic and treatment of tumours and cancer
3.	*Mentha citrata*	0.5-0.8% oil; 45-50% linalool, 35-40% linalyl acetate, carvone (in traces)	The leaves are mixed with orange juice to make a conserve. It is also used in the cosmetic industry
4.	*Mentha longifolia*	1.2% oil; 50% piperitone oxide, 20% piperitenone, diosphenol, diosphenolene (in traces)	Treatment of nausea, gastralgia, neuralgia, rheumatism and headache
5.	*Mentha piperita*	0.3-1% oil; 50-55% menthol, 20% menthyl acetate, 10% menthone, limonene, cadinene, cineole, lactone, terpinele, isovaleric acid (in traces)	The oil is used in dental creams, toothpastes, mouthwashes, cough syrups, chewing gums, beverages and confectioneries. It posses antiseptic and carminative properties. Tea made from leaves and stem is drunk as a relief for indigestion. Peppermint oil has been used as a folk medicine, as a local anaesthetic and anti-spasmodic, and for controlling diarrhea, tooth ache, headache, nausea and cramps
6.	*Mentha pulegium*	1-2% oil; 85-95% pulegone, 4-5% menthol, menthone, menthyl acetate (in traces)	The oil is used for scenting soaps. Pulegone is one of the major components of the oil is a convenient base for the manufacture of synthetic menthol. Strong infusion of leaves is applied to the skin to keep away gnats in summer. It is also used in nervous disorders and as insect repellent
7.	*Mentha rotuntifolia*	0.06-0.1% oil; 51% piperitenone oxide, 10% dipentene, 10% limonene, 15% dihydrocarveol acetate, 15% carvone	Has digestive properties and used in confectionery and flavouring food
8.	*Mentha spicata*	0.2-0.5% oil; 58% carvone, 8% limonene, 10% dipentene, 7% dihydrocarveol, 12% dihydrocarveol acetate	Used in foods, beverages, toothpaste, mouthwashes, soaps, detergents, creams, lotions and perfumes and medicinally as stimulant, carminative, anti-spasmodic and in bronchitis and fever

Uses of mint herbage and essential oils

Mints are extensively cultivated for their oils and terpenoid components of the oil, such as menthol, carvone, linalyl acetate and linalool, for use in pharmaceutical, cosmetic, food, flavour, beverage and allied industries (Chaddha and Gupta, 1995). Table 1 gives the various applications of important mint species.

Genetic information in terpene biosynthesis in mints

The genetics of biosynthesis of chemical constituents of all the important species of *Mentha* has been studied (Hefendehl and Murray, 1972). Utilizing crosses between *Mentha* species, it has been shown that single Mendelian gene(s) control presence or absence of major compounds such as carvone, menthone, menthol and piperitenone/ piperitone. Hefendehl and Murray (1972) suggested that from the biogenetic viewpoint, *M. arvensis* and *M. piperita* perform the same conversions; pulegone→menthone→ menthol→menthyl acetate, except for the slower conversion of menthone to menthol in *M. piperita*. The monogenic basis for the conversion of menthone to menthol showed that gene *R*, either in homozygous (*RR*) or in heterozygous (*Rr*) form, was responsible for the reduction of menthone to menthol or carvone to carveol (Hefendehl and Murray, 1973). The dominant *A* gene allowed the conversion of piperitenone into pulegone. The gene *P* either in homozygous dominant (*PP*) or in heterozygous condition (*Pp*) caused the conversion of pulegone into menthone. It may be pointed out here that gene *P* is controlled indirectly by the gene *F*, responsible for the conversion of pulegone into menthofuran. There is so far no evidence of linkage between these genes (Hefendehl and Murray, 1972).

In M. *spicata* the dominant gene *C* was responsible for production of carvone from limonene, while the dominant gene *Lm* prevented the conversion of limonene to piperitenone. There is little conversion of carvone into its alcohol carveol since the species does not have the dominant gene *R* that allows this conversion. Hefendehl and Murray (1972) concluded that a genotype having dominant *Lm* and recessive *cc* resulted in accumulation of limonene whereas the genotype with both genes in the recessive form (*lm, lm ,cc*) contained only 3-oxygenated compound and no 2-oxygenated compound. The complementary genotype, possessing both dominant genes (*Lm, Lm, CC*), produced only the 2-oxygenated compound, carvone (Murray and Hefendehl, 1973).

The lavender odour of *M. citrata* is caused by 84 to 90% linalool and linalyl acetate. Chemical and genetic analysis revealed that the dominant gene *I* in *M. citrata* resulted in production of 60-90% linalool/linalyl acetate but only 1-1.5% isopinocamphone and 0.5-2.5% α-pinene. All individuals of linalool chemotype must have one dominant *I* gene to produce linalool and 4 dominants are possible since this gene is present on two different homologous pairs of chromosomes.

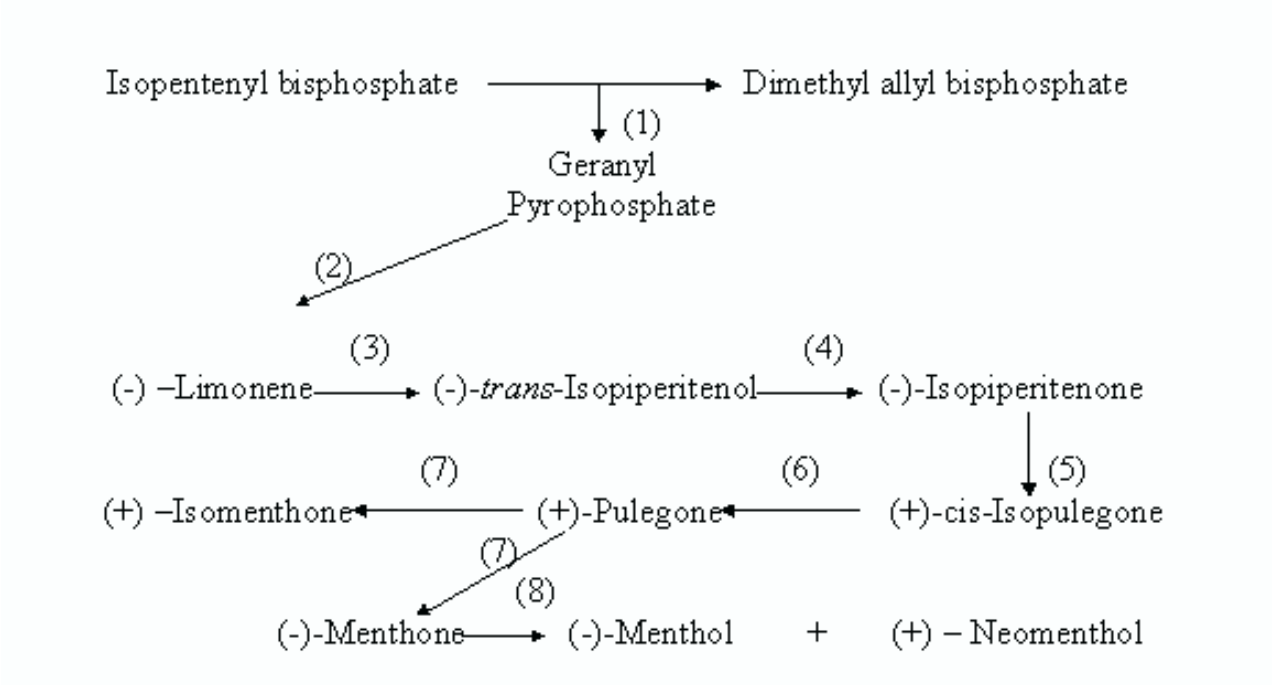

Figure 1. The principal pathway for monoterpene biosynthesis in mint. The responsible enzymes are (1) geranyl bisphosphate synthase, (2) limonene synthase, (3) cytP450 limonene 3-hydroxylase, (4) *trans*-isopiperitenol dehydrogenase, (5) isopiperitenone reductase, (6) *cis*-isopulegone isomerase, (7) pulegone reductase, (8) menthone reductase (modified from Croteau and Gershenzon, 1994; McConkey *et al.,* 2000).

Enzymes involved in monoterpene biosynthesis

The pathway of monoterpene biosynthesis in mint has been well established by *in vivo* and cell-free studies, and all of the enzymes involved have been described (Croteau and Venkatchalam, 1986; Colby *et al*., 1993). Monoterpene biosynthesis and accumulation has been specifically localized to the glandular trichomes (McCaskill *et al*., 1992). The physiology and carbon metabolism of glandular cells are best studied in mint (McCaskil and Croteau, 1999). The pathway originates in the plastids (leucoplasts) of the secretary cells of these highly specialized non-photosynthetic glandular structures (Turner *et al*., 1990). The monoterpene family of natural products therefore is derived from the plastidial, mevalonate-independent pathway for isoprenoid metabolism, which provides isopentenyl bisphosphate (and, by isomerization, dimethylallyl bisphosphate) as the universal precursor of the terpenoids. Aspects of monoterpene production in callus cultures of mint also have been studied in relation to its production in whole plant. A recently discovered pathway and the enzymes involved in monoterpene biosynthesis in the mint is summarized in Figure 1. The oxygenation pattern of monoterpenoids of mint has been determined by regiospecific cytochrome P450-catalysed hydroxylation of the common olefinic precursor, (-) limonene. A PCR product of limonene-0-6-hydroxylase was used as a probe to isolate full length cDNA clone which provided the tool for isolating the homologous cDNA from peppermint and related *Mentha* species (Lupien *et al*, 1995).

Pests and Pathogens of mints

Mint crops are associated with large numbers of pests and pathogens that cause substantial damage to crops and considerable loss of oil yield. Termites attack the underground parts and cause serious damage to the root system and main stem of the plants. Cutworms damage the young shoots of mint plants after they have sprouted. Polyphagous caterpillars often cause severe damage to the crop by defoliating it within a few days. The polyphagous semiloopers also voraciously devour the tender foliage. White flies lay eggs on the ventral surface of the mint leaves, and the nymphs suck the sap resulting in arrested growth.

A number of microbes have been observed to cause diseases in mint crops. The stolon rot that occurs in the rainy season is caused by *Macrophomia phaseoli* and *Pythium*. In the affected plants, leaves wilt and turn yellow and growth is stunted. Rust is caused by *Puccinia menthae;* orange colour rust postules appear on leaves, which afterwards turn yellow and ultimately get shed. The powdery mildew caused by *Erysiphe cichoracearum* results in circular white powdery patches on the leaves, which subsequently spread to the stem and other parts of the plant; the severely affected leaves drop off. Leaf blight caused by *Alternaria* species during summer months results in the appearance of irregular dark brown spots with concentric zones surrounded by a pale yellow margin on the upper surface of the leaves; heavy defoliation occurs subsequently. The leaf spots caused by *Corynespora* spp. also inflict considerable damage on mint crops (Shukla *et al*., 1999).

Several types of control measures, including the use of insecticides and disease-inhibiting chemicals, and breeding of pest and pathogen resistant cultivars have been followed in mints but limited success has been obtained in controlling both pest and pathogens. There is a need for genetically engineered important mint cultivars with heterologous genes for rendering them pest or disease resistant.

Conventional pesticides used on mint crops

Applications of gondal mixtures (gardenia gummifera, asafoetida, aloes, rosin in the ratio of 1:2:2:2 diluted in water) and insecticides like chlordane, dieldrin, heptachlor and toxaphene have been recommended for controlling termites and must be applied at 3 stages; before sowing, at planting, or after planting of suckers. Soil application of aldex or chlorodane before planting or spraying with metasystox, malathion or thiometan gives effective control against cutworms. For the early stage attack of caterpillars, 5% diptrex or 2% folidol, and for advanced stage, spraying with thiodon or endrin gives effective control. Semilooper infection can be controlled by spraying with endosulfan or carbayl mixed in water. Leaves badly infested with white flies have to be collected and burnt or the crops sprayed with rosin compounds to destroy the insects. Waterlogging promotes infection of pathogens, viz. *Macrophomia* and *Pythium*. This can be avoided by planting *Mentha* on ridges. The treatment of planting stock with fungicides effectively controls the pathogens. Hot water treatment, by steeping the stolons in water or spraying with copper fungicide (0.3% containing 50% metallic copper) or sulfur powder (0.5% containing 50% elemental sulfur) in water, controls rust. Powdery mildew disease can be controlled by spraying with copper fungicides and lime sulfur. Spraying of copper fungicide also helps in checking blight disease caused by *Alternaria*.

Breeding approaches for deriving disease and/or pest resistant mint cultivars

Various breeding approaches such as intra- and inter-specific hybridizations, clonal selection, polyploidy and induced mutagenesis have been employed for evolving genetically improved genotypes of *Mentha* for use in breeding of disease and pest resistant cultivars. With few exceptions, polyploidy increases the resistance to rust in Japanese as well as Canadian forms of *M. arvensis*. Resistance to *Verticillium* wilt has been observed in the Bulgarian mint variety. The varieties such as MAS-1 and Kalka of *M. arvensis* have been shown to possess resistance to rot and rust diseases. The variety EC-41911, developed by progeny selection in the interspecific cross between *M. arvensis* and *M. piperita*, has been observed to be less susceptible to aphids. Following irradiation with x-rays and neutrons, *M. piperita* strains resistant to *Verticillium arboretum* have been isolated. *M. piperita* (2n=144) and *M. spicata* (2n=48) were crossed to obtain

hybrids (2n=96) that were resistant to both frost and rust. A liquid suspension of microconidia was injected into the roots and surrounding soil of *M. piperita* and *M. spicata* to develop resistance against *Verticillium dahlia* (Sink and Grey, 1999).

Pest and disease control in mint crops has been achieved with limited success. The polyploid nature of the crop results in limited recovery of resistant mutants. Further controlled pollination to develop hybrids for recovering plants of interest in further generations is limited due to the small size of the flowers which occur in clusters, making emasculation difficult and viability of seeds poor (Chaddha and Gupta, 1995). Despite the undoubted success of chemical insecticides, a number of problems have become apparent in terms of their harmful effects on human health, environmental pollution and ecological imbalance.

Apparently, one of the alternative strategies of mint crop protection is the use of biopesticides. A number of strategies including the use of genes encoding for delta-endotoxins, protease inhibitors, lectins and amylase inhibitors are being tried for control of insects in a number of economically important crops. Amongst insecticidal genes, the *delta-endotoxin* gene of *Bacillus thuringiensis* (Bt) can be considered of major significance in controlling insect pests of mints.

Currently, 95% of the commercial biopesticides are derived from *B. thuringiensis*. Formulations of *B. thuringiensis* spore crystal sprays, such as dipel and thuricide, are commercially available for use as biological insecticides. The advantages of biopesticides over chemical insecticides are high specificity and safety to non-target organisms and beneficial insects. These types of biopesticides should be exploited in mints since mints are industrially processed before their products find use.

Bacillus thuringiensis bacterium is a natural insecticide

B. thuringiensis is a gram positive spore forming bacterium that exists in soil and grain storage dust and on plant surfaces (Ruud *et al*., 1999). *B. thuringiensis* produces the insecticidal crystal proteins (ICPs) crystal (cry) and cytolytic (cyt) which are selective for several types of invertebrates. ICP-encapsulated systems are created in order to increase the persistance and stability of toxins under field conditions (Priesto *et al*, 1997). The different crystal protein genes *(cry*) in respective *Bt* strains, presumed to be responsible for production of specific pro-toxins, are classified into *cry*I to IV groups (Hofte and Whiteley, 1989). Recently, two new classes, viz. *cry* V and *cry*VI, which are nematicidal in nature, have been added to these groups .

Mode(s) of action(s) of cry endotoxin proteins

Electro-physiological evidence suggested that crystal proteins are dissolved in the larval insect midgut (pH 9 - 10) and are proteolytically converted into toxic core fragments. The toxins induce the formation of small, nonspecific pores (0.5 - 1nm in size) in the membrane of epithelial cells of susceptible insects, resulting in a net influx of ions accompanied by inflow of water. As a result, the cells of the insect gut swell and are lysed. Biochemical studies of isolated midgut membrane concluded differently. K^+-amino acid co-transport into BBMV (brush border membrane vesicle) prepared from midguts of *Pieris brassicae* larvae has been studied and it is observed that treatment with toxin immediately inhibited the transport of labeled histidine driven by a K^+ gradient. As there was no influence of toxin on the K^+-amino acid co-transport itself, the K^+ gradient was considered to be dissipated through the formation of channels in the BBMV upon toxin treatment. Amino acid transport in this tissue can also be driven by a Na^+ gradient although less efficiently.

Benefits of cry+ mint crops over cry (or Bt) sprayed mint crops

Cry protein can protect mint crops from insects if sprayed with active or inactive Bt spores. However, the high cost of production and the need for repeated applications due to poor persistence of sprayed material under field conditions disfavour such an approach. The alternative available strategy of plant protection is to incorporate insecticidal genes into mint crops by genetic engineering.

A number of insecticidal genes of different origin have been introduced to develop resistance against insects in different crops, but none of these genes have been introduced into any of the mint species. It appears promising to develop mint cultivars bearing insect resistance giving one or more cry or plant derived genes.

Current status of plant transformation technology

Various requirements for the introduction of transgenes into the desired plant species for the development of stable fertile transgenic plants are: 1. Efficient tissue culture process(es) for shoot regeneration and/or propagation; 2. Efficient DNA delivery process; 3. T-DNA integration method(s); 4. Selection strategy for transgenic tissue; 5. Strategy for the expression of transgenes.

Shoot regeneration and/or propagation by the tissue culture process

Advances in tissue culture, combined with improvements in transformation technology have resulted in increased transformation efficiencies. A tissue culture stage is required in most current transformation protocols to recover plants and it is the totipotency of plant cells that underlies most plant transformation systems. The regeneration of plants in *in vitro* is controlled by plant growth regulators and other factors added to the culture medium, tissue type, light and temperature.

Role of plant growth regulators in *in vitro* cultures

Plant growth regulators (PGR) are one of the most important factors which control growth and differentiation of tisssue(s) in *in vitro* cultures. Among PGRs, auxin and cytokinin singly or in combination play effective roles in inducing growth in plant cultures. Auxin exerts a strong influence on the organization of meristems giving rise to

either unorganized tissue (callus) or defined organs (roots) and promotion of vascular differentiation (Davies, 1995). The commonly used auxins in tissue culture are 2,4 dichlorophenoxy acetic acid (2,4D), 1-naphthalene acetic acid (NAA) and indole compounds such as indole acetic acid (IAA) and indole butyric acid (IBA). Several other indole derivatives, both natural and synthetic viz. indole-3-acetaldehyde, indole-3-acetamide, indole-3-acetonitrile, indole-3-acrylic acid, indole-3-lactic acid, indole-3-propionic acid and indole-3-pyruvic acid are active in cultures. Among others, 3,6-dichloro anisic acid (dicambra) and 4- amino 3,5,6 trichloropyridine-2-carboxylic acid (picloram) are often effective in inducing the formation of embryogenic tissue or in maintaining suspension cultures (Hagen *et al*., 1991).

The properties of cytokinins that are useful in culture are the stimulation of cell division and dedifferentiation. They can thus induce adventitious bud formation (Krikorian, 1995). The most commonly used cytokinins in plant tissue culture are zeatin, 2-isopentenyl adenine (2iP), dihydro zeatin, zeatin riboside and the purine substitutes, kinetin and benzyl amino purine (BAP). Adenine, adenosine and adenylic acid also have cytokinin activity, but less than that of the commonly used cytokinins. The exposure of plant tissue to thidiazuron (TDZ) for relatively short periods is sufficient to stimulate regeneration.

In vitro Plant regeneration methodology

Plants are regenerated from cell culture via two methods, somatic embryogenesis and organogenesis. Proliferating somatic embryos in liquid culture or on solid medium are suitable targets for transformation because the origin of proliferating embryogenic tissues is at or near the surface of the older embryos and thus readily accessible to DNA delivery (Hansen and Wright, 1999). Embryogenic tissues are, in general, very prolific and allow recovery of many transformants that are, in most cases, non-chimeric because of the assumed single cell origin of somatic embryos.

Organogenesis is the generation of organs, usually shoots, from a variety of tissues like the stem and leaf segments, cotyledon, hypocotyl and scutella from embryos (Schopke *et al*., 1996; Li *et al*., 1996). The advantage of this system over using somatic embryos is that shoots can usually form roots readily. If the roots fail to appear, grafting can be the solution and the process aids in recovering of plants in a short period. Different species of *Mentha* have been cultured in various media prepared by combining various PGRs with varied objectives, including shoot regeneration and micropropagation.

Progress in the work on *in vitro* culture in *Mentha* species

Different media such as White's, Murashige and Skoog's and Gamborg's have been used alone or in combination with various PGRs like 2,4D NAA, IAA, BAP and kinetin to develop and standardize culture conditions for morphogenesis or for obtaining undifferentiated mass of callus tissue in mint. The effects of various growth factors on developing callus in solid medium or cell suspension cultures have been investigated to obtain the desired terpene component, but little effort has been made to obtain either direct or indirect shoot regenerants from various explants, which has been a prerequisite in transformation technology. However, embryonic suspension and callus cultures were used initially and recent results showed that the primary explants seem to be more advantageous for the routine production of fertile transgenic cereals (Jaehne *et al*., 1995). The conditions standardized for obtaining regenerants from various explants *in vitro* in *Mentha* species are presented in Table 2.

The DNA delivery process

Successful plant transformation, in addition to a reproducible tissue culture technique, requires an efficient DNA delivery method. In plant transformation technology, besides entire explants, the isolated protoplasts can be transformed by *Agrobacterium* or by direct DNA uptake methods, facilitated by polyethylene glycol treatment, electroporation or liposomes (Shillito, 1999). Other methods, such as direct and indirect gene transfer, microinjection, laser microbeam, chemical treatments, particle bombardment (biolistic method) are commonly used (Thomzik, 1996). Plants can be recovered after DNA delivery using silicon carbide whiskers (Thompson *et al*., 1995). Microinjecting DNA into zygotes is an alternative method that could eventually lead to recovery of transgenic plants (Leduc *et al*., 1996). The choice of technique for DNA delivery depends on convenience, tissue accessibility, patent clearance and equipment availability.

Process of *Agrobacterium tumefaciens* mediated DNA delivery

The natural ability of the soil microorganism *Agrobacterium* to transform plants is exploited in the *Agrobacterium*-mediated transformation method. Transgenic plants obtained by this method often contain single copy insertions (Hansen and Wright, 1999). The virulent strains of *A. tumefaciens* carry one or more extra chromosomal element(s), plasmid(s), that harbour genes for biological functions concerned with plasmid maintenance, bacterial adaptation and tumour induction in plants.

Agrobacterial *virulence* genes

The genes *virA, virB, virD* and *virG* are known to be essential for T-DNA transfer while others are host range genes and affect the efficiency with which *Agrobacterium* transfers T-DNA among different plant species. Besides the virulence genes residing on the Ti-plasmid, genes coding for products effecting the virulence of bacterium and essential for early plant-*Agrobacterium* interaction are present on the chromosome and termed chromosomal virulence (*chv*) genes; namely *chv*A (1.5Kb), *chv*B (5Kb) and *pscA or exoc* (3Kb) (Cangelosi *et al*., 1987).

Table 2: Culture conditions for axillary proliferation and adventitious regeneration from different types of explants in *Mentha* species

Sl no	Nature of explant mate-rial	*Mentha* species	PGR(s) and their conce-ntration (mg/l)	Type of regener-ation response	Degree of proliferation in explant	Remark(s)
1	Node	*M. spicata*	BAP (1 or 2) and KIN (2) on MS basal medium	Direct axillary prolifera-tion	15 shoots in 40 days	Roots were formed almost at the same time as shoot proliferation, occurred from axillary buds.
		M.piperita	-do-	-do-	20 shoots in 40 days	-do-
		M. viridis	-do-	-do-	15 shoots in 40 days	-do-
		M. arvensis	-do-	-do-	1 or 2 shoots in 45 days	-do-
2		*M. piperita*	2,4D + KIN in MS basal medium	-do-	4 shoots in 20 days	Soaking of explant for 3-4 h. in double distilled water facilitated dilution of the oxidised phenolics; addition of activated charcoal played a vital role in regeneration
3		*M. arvensis*	BAP (3) +IAA (1) in MS medium	Axillary bud prolifera-tion via callusing	30-40 shoots in 8 weeks / 60 days	Somaclonal variation was observed among regenerants
4	Inter-node	*M. arvensis cv. Kalka*	BAP (5 or 10) + NAA (1 or 2) in MS medium	Regeneration of shoots via callusing	179 shoots in 12 weeks	The RAPD profiles of invitro raised plantlets of cv. Kalka were identical to the parent
		cv Himalaya	-do-	-do-	205 shoots in 12 weeks	The frequency of polymorphic bands in the regenerants was 3.6%
5	Shoot tip	*M. spicata*	BAP (0.2 or 2) +NAA (0.02 or 0.2) in B5 medium	Production of plantlet via callusing	Several in 180 days	Somaclonal variation was observed
6	Leaf disk	*M. citrata*	BA (8) and coconut water in MS media with CW	Shoot regeneration via callus formation	6 shoots per disk	Higher regeneration in the presence of coconut water and cultures incubated under dark
7	Leaf disks	*M. piperita*	NAA (0.1) BA (1.5) and TDZ (0.1) in MS medium	Shoot regeneration via callusing	78% regeneration at 6 weeks	Mannitol in addition to sucrose was used in the medium untill the initiation of callusing was achieved
		M. spicata	-do-	-do-	49% regeneration at 6 weeks	-do-

-do- indicates same as above

Activation process for the *vir* genes of *Agrobacterium*

As compared to *chv* genes which are constitutively expressed, *vir* genes remain silent until induced by certain plant factors. These plant factors from tobacco have been identified as the phenolic compounds acetosyringone and α-hydroxy acetosyringone (Stachel *et al.*,1985). It has been shown that other phenolic compounds, including lignin precursors such as coniferyl alcohol and sinapic acid, can also act as virulence inducers (Bolton *et al.*, 1986).

The virulence genes, *virA* and *virG*, act as a two component regulatory system for the induction of other virulence genes (Lee *et al.*, 1996). The complex formed by sugars released via wounding and *chv*E, a periplasmic glucose-galactose binding protein, interact with the periplasmic domain of Vir A protein (Shimoda *et al.*, 1993) and the phenolic compounds interact directly with the periplasmic domains of Vir A proteins (Lee *et al.*, 1996). These interactions lead to the autophosphorylation of histidine residue in the kinase domain of Vir A protein and transfer PO_4^{-3} to asparagine residue of Vir G protein (Turk *et al.*, 1994; Lee *et al.*, 1996). This activates Vir G protein which in turn binds to the promoters of different *vir* genes to further induce the process (Rogowsky *et al.*, 1987).

Transfer of T-DNA from *Agrobacterium* to plant cells

The *vir* D gene(s) encode 4 polypeptides. The two polypeptides VirD1 and D2 are required for the endonucleolytic cleavage at the T-DNA borders (Vogel and Das, 1992). During its transit the T-strand is associated with 2 protein products of the *vir* genes, VirD2 and VirE2. After the transfer of T-complex to the plant cell, plant receptor such as galacturonan (Rao *et al.*, 1982) or pectin binds to the complex (Neff *et al.*, 1987). The diagrammatic representation indicating induction of virulence genes, T-DNA processing and its movement is shown in Figure 2.

Vector systems based on *Agrobacterium* technology

Besides T-DNA, no other part of the Ti-plasmid becomes integrated into the genome of the plant cell via *Agrobacterium*. On this basis, vector system for the transformation of plants have been developed and distinguished into *cis* and *trans* type. To widen the application of *Agrobacterium* mediated plant transformation, highly efficient vectors were designed with extra copies of the *vir* genes (super binary vectors) or with the mutations that enhance *vir* gene expression (Hansen and Chilton, 1999).

Genetic markers and facilitating agents for the selection of transformants in plants

Transgenic plant cells carrying wild type Ti-T-DNA (oncogenes) are tumorous and can not be regenerated into plants. However, plant cells transformed with disarmed or non-oncogenic T-DNA behave in the same way as untransformed plant cells of the same species in tissue culture. As transformed plants are nearly indistinguishable from non-transformed ones, markers to detect transformed cells/plants have been developed which make their carriers relatively resistant to specific antibiotics and/or herbicides. Sometimes, reporter genes that will co-express with the gene of interest are used in place of selectable markers. Vectors are now available which allow selection of transformed cells/tissue/organ via selection or reporter marker genes.

Occurrence of transgene silencing

The production of transgenic plants for a number of crop plants will only be of value if the engineered phenotype is faithfully transmitted through subsequent generations in a predictable manner. Levels of transgene expression in plants are generally unpredictable and vary between independent transformants; this variation is usually explained in terms of differences in transgene copy number and/or integration site (Prols and Meyer, 1992). The instability of transgene expression can be observed within a single generation (Meyer *et al.*, 1992). It was established at the molecular level that the expression is not correlated with the loss of the transgene, but rather with its inactivation (Scheid *et al.*, 1991). Inactivation of T-DNA was first observed in *A. tumefaciens* induced tumor lines that contained multiple copies of the introduced T-DNA (Gelvin *et al.*, 1983).

The introduction of foreign genes by T-DNA mediated transformation is not itself responsible for transgene recognition and inactivation, as DNA introduced by direct gene transfer methods is also subject to inactivation. Inactivation occurred more frequently when the gene was present in multiple copies suggesting that this phenomenon be termed RIGS (repeat induced gene silencing) (Assaad *et al.*, 1993). Although single copy transgenes can be inactivated, transgene inactivation generally occurs at highest frequency when multiple copies of the gene are integrated either at a single insertion site or dispersed throughout the genome (Ottaviani *et al.*, 1993). A common goal of efforts in plant biotechnology is the over-expression of an endogenous or introduced trait. Introduction of extra copies of an endogenous gene to boost expression may, however, result in the co-ordinate silencing not only of the introduced transgenes but also of the endogenous gene(s). This phenomenon, termed co-suppression, has been described in the *Petunia* chalcone synthase (*chs*) gene (Hobbs *et al.*, 1993). Newly integrated DNA, whether it be a transposed mobile element or an introduced transgene, may be recognized as foreign because its insertion disrupts normal chromatin structure, or alternatively sequence characteristics of the integrated DNA could distinguish it from that of the surrounding integration site. Plant genomes are mosaics of compositionally homogeneous DNA segments with defined GC contents, termed as isochores. Disruption of the normal make-up of an isochore by insertion of DNA that differs in GC content may mark this region for inactivation and methylation (Meyer and Heidmann, 1994).

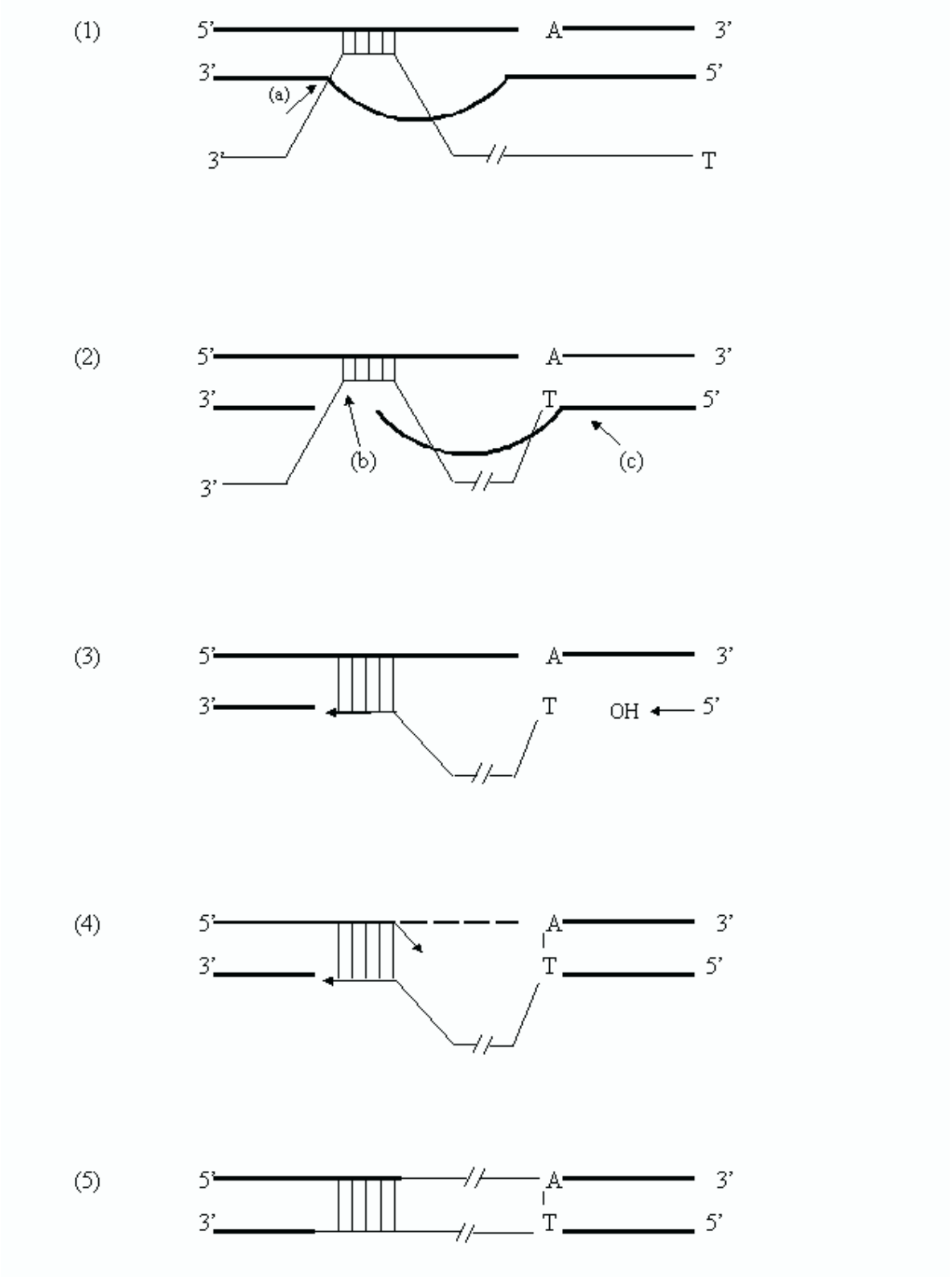

Figure 2. Model for donated T-DNA integration into host DNA. The host plant DNA and T-strand are respresented by thick and thin lines respectively. (a), (b), (c), and (d) indicate the possible sites for endonucleolytic attack. Arrow indicates DNA replication and dashed lines represent DNA strand subjected to nucleolytic degradation (modified from Tinland *et al.,* 1995).

Variability in expression of *Bt* gene in plants

The feasibility of raising insect-resistant transgenic lines of agricultural and horticultural crop plants through transfer and expression of crystal protein genes of *B. thuringiensis* has been demonstrated. Successful attempts for insect control in tobacco plants engineered with crystal protein genes have been made. Transgenic tomato plants resistant to tobacco hornworm, budworm and corn earworm with a full length 3.4 Kb as well as truncated 1.9 Kb fragment of *cry* IA (b) gene under CaMV 35S promoter have been raised (Fischoff *et al.*, 1987). Transgenic tomato plants expressing *cry* 1A(b) gene on evaluation of performance in field showed very limited feeding damage to the leaves against *Maduca sexta* (Delannay *et al.*, 1989). Significant control of *Heliothis virescens* and *H. zea* was also observed.

The expression of native (unmodified) *cry* genes has been examined in transgenic tobacco, tomato, potato, carrot and cotton plants, which revealed poor expression of the gene and low levels of larvicidal polypeptide (Perlak *et al.*, 1990; Murray *et al.*, 1991; Cheng *et al.*, 1992). The low levels of cry proteins in higher plants have been attributed to several factors, including (the lack of) an efficient vector, appropriate promoter, leader sequences, 3' non-coding sequences, plant regulator sequences, codon frequency, the secondary structure of the mRNA and the gene product itself (Perlak *et al.*, 1990, 1993). Among the various factors perhaps the stability of transcribed *cry* mRNA in higher plants is the major limitation. This has been attributed to the inefficient use of prokaryotic AT-rich codons by the transcriptional machinery of plants (Murray *et al.*, 1991; Aarssen *et al.*, 1995).

Because plant systems prefer GC-rich codons, inefficient expression of prokaryotic genes within the eukaryotic environment reflects differential codon usage. This has necessitated the modification of *cry* genes in plants with stable protein expression. Over time, highly modified genes with plant preferred codons and also completely synthetic genes have become available for efficient expression in plants (Wunn *et al.*, 1996; Nayak *et al.*, 1997). Enhanced gene expression appears to be the result of increased translational efficiency of the prokaryotic gene in eukaryotic cell. Expression of modified *cry* genes has also been checked along with different efficient plant promoter and leader sequences (Wong *et al.*, 1992). The *ats* 1A promoter with its transit peptide sequence and 5' untranslated leader fused to *cry* 1A (c) gene provided a 10-20 fold increased expression of the cry protein compared with the CaMV 35S promoter with duplication of the enhancer region. The alternative approach has been the incorporation of leader sequences with *cry* gene for targeted expression into the chloroplast of higher plants (McBride *et al.*, 1985). It has been feasible to breed new cultivars with multiple resistances via genetic engineering and raise virus resistant and insect tolerant transgenic tobacco plants (Liang *et al.*, 1994).

Progress in genetic transformation experimentation in certain commercial crops

Transgenic plants have been produced in more than 90 plant species and a series of marker genes, including several agronomically important genes for herbicide resistance, insect and disease resistance have been incorporated through various transformation methods (Brar *et al.*, 1995). A number of economically important crops, viz. cassava (Li *et al.*, 1996), sugarbeet (Lindsay and Gallois, 1990), cucumber (Nishibayashi *et al.*, 1996), pea (Jordon and Hobbs, 1993), sweet potato (Gama *et al.*, 1996), *Brassica oleraceae* (Srivastava *et al.*, 1988), *Brassica rapa* (Kuvshinov *et al.*, 1999), rapeseed (Fukuoka *et al.*, 1998), tomato (Jacinto *et al.*, 1997), potato (Surov *et al.*, 1998; Lecardonnel *et al.*, 1999) and many more have been transformed via *Agrobacterium tumefaciens* and/or microprojectile bombardment. It is noteworthy that in all cases, tissue culture steps were involved for obtaining transgenics. Success in obtaining transgenics in the mint crops has been limited. Mostly marker genes have been transformed in *Mentha* plants using *Agrobacterium tumefaciens* mediated system. Beta-glucuronidase (*gus* gene) and neomycin phosphotransferase (*npt* II gene) have been transformed using particle bombardment (Caissard *et al.*, 1996; Niu *et al.*, 1998, 2000). The authors are engaged in developing *cry*$^+$ transgenic *M. arvensis* plants. The parameters, e.g. DNA donor *Agrobacterium* strains, acetosyringone concentration, conditions of transformation, and selection are being standardized. The optimization of conditions for microprojectile bombardment mediated transfer is also being carried out.

Conclusion

Mentha species are resources for essential oils enriched in certain monoterpenes and are widely used in the food, flavour, cosmetic and pharmaceutical industries. Mentha has a large number of species differing in their characteristics and polyploidy level. Crosses between Mentha species showed that single Mendelian gene(s) control the presence or absence of major compounds like carvone, menthone, menthol, piperitone etc. Genetic information and enzymes involved in monoterpene biosynthesis are reviewed. Pests and pathogens cause substantial damage to the crop and also considerable loss of oil yield. Termites, cutworms, whitefly and semiloopers damage different parts of the *Mentha* plant. Microbes, viz. *Macrophomia phaseoli* and *Puccinia menthae* also damage the *Mentha* plant. Insecticides and disease-inhibiting chemicals are used in the control of these conditions. Nowadays, experiments are in progress for the successful transformation of pest resistant gene(s) using protoplast and subsequent tissue culture techniques.

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