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EXPLORING BIODIVERSITY TO ENHANCE BIOACTIVITY IN THE GENUS *TANACETUM* THROUGH PROTOPLAST FUSION

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ABSTRACT

Tansy (*Tanacetum vulgare* L.), which is a wild and under exploited plant species adapted to Northern climate and characterized by its bioactivity against insects mediated by isoprenoids was the focus of this thesis research. The disadvantage of tansy isoprenoids for further utilization is that they exist at a low concentration in the plant and they are not as active against insects as the group of related isoprenoids, pyrethrins. Pyrethrins are produced by another *Tanacetum* species, pyrethrum (*Tanacetum cinerariifolium (Trevir.) Schultz-Bip.*), which, however, does not tolerate long and cold winters. Pyrethrins are environmentally benign insecticidal compounds with an increasing world market demand.

The overall goal of this thesis was to explore the biodiversity of Finnish tansy and utilize it in a protoplast fusion program when prospecting towards enhanced bioactivity incorporated into a new interspecific crop species adapted to Nordic climate. The specific aims were to: 1) detect genetic, chemical and morphological variation of tansy; 2) develop *in vitro* conditions for tansy and pyrethrum suitable for micropropagation and for donor tissue production to be used in protoplast isolation; and 3) develop protoplast isolation and chemical fusion methods for tansy and pyrethrum and to test the hybridity.

All of the 20 tansy genotypes included in this study differed genetically as analyzed by RAPD-PCR; in the composition of volatiles analyzed from flower heads of tansy by GC-MS; and in their selected morphological characters. Linkage between genetic, chemical, and morphological characters and the origin of the chemotype was observed. The RAPD-PCR patterns clustered the tansy lines into two groups corresponding to climatic and geographic zones in Finland. In total, 55 volatile compounds were detected which grouped the 20 tansy lines into eight chemical clusters. Irregular monoterpene, davanone, was detected from tansy grown in Finland for the first time. Additionally, three other chemotypes accumulating artemisia ketone, which is another irregular monoterpene, was identified.

Tansy turned out to be a recalcitrant species in tissue culture. Tansy lines contained endophytic bacteria which was problematic when transferring shoot tips to *in vitro*. Other symptoms such as tissue browning and hyperhydricity contributed to the recalcitrance of tansy. These adverse symptoms were partially overcome by optimizing the growth conditions for tansy. Growth conditions such as low light intensity during the culture of tansy and pyrethrum for protoplast isolation experiments increased the frequency of successful protoplast isolation and protoplast-derived callus formation. Especially protoplast isolations carried out between February and March resulted in protoplast-derived callus growth in tansy. The observed regeneration from explants as well as spontaneous root and shoot formation from protoplast-derived callus shows that tansy has the ability to regenerate in *in vitro* culture.

Chemical fusion method between tansy and pyrethrum protoplasts was developed. Three callus clones of which one was derived from an intraspecific fusion of tansy (tansy x tansy) and two from an interspecific fusion between tansy and pyrethrum were successfully tested for their hybridity already at the callus level. All the tested fusion calli had usually higher nuclear DNA content than either of the parental tissues suggesting that fusion between at least two protoplasts had occurred. The RAPD-PCR patterns of the

putative fusion callus clustered the callus derived from an intraspecific fusion closer to tansy than to pyrethrum whereas calli derived from an interspecific fusion was positioned closer to pyrethrum than to tansy. Thus, somatic hybridization between tansy and pyrethrum had occurred, although most of the volatiles detected from the fusion calli were not detected from the intact plants. The absence of terpenes typical for donor tissues may not be due to absence of hybridization but rather an indicator of the ability of unorganized callus tissue to accumulate species specific compounds.

Based on the results it can be concluded that tansy grown in Finland contains a wide range of genetic variation. Especially, among the large number of volatile compounds detected from tansy, the chemotypes accumulating irregular monoterpenes which are biochemically close to pyrethrins are an interesting source for biotechnological applications such as protoplast fusion and could impart economical value. In addition to volatile compounds, also less volatile sesquiterpenes and flavonoids could be used as a source for further investigations related, not only to insecticidal activity, but also medicinal properties. The observed interesting variation can be enhanced and further utilized by combining it with another species. Protoplast fusion method which has been used for the improvement of many important agronomic plant species can also be a novel tool for metabolic engineering. The un-necessity of knowledge of specific biochemical steps required in genetic engineering is the most important advantage using protoplast fusion method for metabolic engineering of wild or less utilized species. Moreover, new interspecific hybrids may combine the enzymology of secondary metabolites in a way that results in production of novel compounds.

LIST OF PUBLICATION

The thesis consists of the following papers, in the text referred to by their Roman numerals.

I **Keskitalo, M., Lindén, A. and Valkonen, J.P.T.** 1998. Genetic and morphological diversity of Fnnish tansy (*Tanacetum vulgare* L.). *Theoretical and Applied Genetics* **96**: 1141-1150.

II **Keskitalo, M., Pehu, E. and Simon, J.** Variation of volatile compounds from tansy (*Tanacetum vulgare* L.) related to genetic and morphological differences of genotypes. *Biochemical Systematics and Ecology* (accepted).

III **Keskitalo, M., Pohto, A., Savela, M-L., Valkonen, J.P.T., Simon, J. and Pehu, E.** 1998. Alterations in growth of tissue-cultured tansy (*Tanacetum vulgare L.)* treated with antibiotics. *Annals of Applied Biology* **133**: 281-296.

IV **Keskitalo, M., Kanerva, T. and Pehu, E.** 1995. Development of *in vitro* procedures for regeneration of petiole and leaf explants and production of protoplast-derived callus in *Tanacetum vulgare* L. (Tansy). *Plant Cell Reports* **14**: 281-296.

V **Keskitalo, M. and Pehu, E.** Improved callus formation from tansy (*Tanacetum vulgare* L.) and pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.] protoplasts (submitted for publication).

VI **Keskitalo, M., Angers, P., Earle, E. and Pehu, E.** 1999. Chemical and genetical characterization of calli derived from somatic hybridization between tansy (*Tanacetum vulgare* L.) and pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.). *Theoretical and Applied Genetics* **98**: 1335-1343.

LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
bp	base pair(s)
CH	casein enzymatic hydrolysate
$CsCl_2$	cesium chloride
2,4-D	dichlorophenoxyacetic acid
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FDA	fluorescein diacetate
FPP	farnesyl pyrophosphate
GA_3	gibberellic acid
GC	gas chromatography
GPP	geranyl pyrophosphate
HMG-CoA	3-hydroxy-3-methyl-glutaryl CoA
HMGR-CoA	3-hydroxy-3-methyl-glutaryl CoA reductase
IBA	indole butyric acid
IPA	6-dimethylallylamino purine
IPP	isopentenyl pyrophosphate
K_I	Kovat's indices
KIN	kinetin
MS	mass spectrometry
MS	Murashige and Skoog medium
NA	nutrient agar
NAA	α-naphthaleneacetic acid
PCA	principal component analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol
RAPD	random amplified polymorphic DNA
R_I	retention indices
Tc	*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.
TSA	tryptic soy agar
Tv	*Tanacetum vulgare* L.
W5	W5-solution

I INTRODUCTION

1. Utilization of wild species prospecting towards new crops

Wild and weedy species contain an enormous source of under-exploited germplasm (Frankel and Bennett 1970). Although, most of the plant species originate from Africa, America or Asia, where the centers of diversity still exist, there are a vast number of under-exploited species also in the Northern Europe (Nordiska Genbanken 1994; Lindberg 1993). Of the identified 250 000 plant species in the global flora, the germ plasm utilized in modern plant production has continuously narrowed. Selection for uniformity and closely defined breeding objectives resulting in pure lines, has led to a marked reduction in genetic variation. On the other hand, very narrow group of plants are dominating current plant production (Frankel and Bennett 1970).

1.1 New crops from under-exploited species

There are several interacting forces which press the public attitude towards new crops: worldwide loss of biodiversity, low prices for major plant production commodities, interest in more sustainable and diversified agriculture, and increasing demand for new foods and plant products (Imrie et al. 1997a; Janick 1996; Janick et al. 1996). Commercially, under-exploited plant derived compounds also attract innovators and entrepreneurs as a source for novel materials. Domestication of a new crop is a process that cannot be achieved in a few years, but if successful the benefits can give a significant impact in many areas (Fletcher 1998; Imrie et al. 1997a; Imrie et al. 1997b; Janick 1996).

Currently in Finland there is increasing interest in alternative crops programs. Several new crops for fibre (canary reed grass, linen, hemp), bioenergy (canary reed grass, rape seed), oil (flax, camelina) and food (buckwheat, spelt, quinoa) are studied in the Agricultural Research Centre of Finland, University of Helsinki, and in food and paper industry. An other group of new crops consists of species containing bioactive compounds. Examples of these are dyes (*Isatis*, *Solidago*), herbs (*Mentha*, *Origanum*, *Thymus*, etc.), medicinal plants (*Catharanthus*, *Echinacea*, *Ginseng*, *Hypericum*, *Solidago*, etc.), health promoting species (*Avena*, *Brassica*, *Fagopyrum*, *Linum*, *Secale*, etc.), and pesticidal (*Anethum*, *Carum, Tanaceum*) crops which produce valuable but neglected or under-exploited secondary metabolites. Finland might have a comparative advantage in finding new bioactive secondary metabolites, as the growing conditions in the north close to the Arctic Circle may promote the production of secondary compounds (Fahlén et al. 1997; Burbott and Loomis 1967).

1.2 Value of new crops

There is increasing demand for new economically viable livelihood options in rural areas of Finland. The most obvious benefit of domestication of a new crop would be the development of processing, packaging, and other value-added activities that would result in job creation and activate local rural communities (Fletcher 1998; Janick et al. 1996).

Because new crops are raised first on relatively small acreages, initial processing can be small-scale, carried out by local entrepreneurs. Although some new crops may substitute for current crops in the marketplace, many new crops will have little or no displacement effect on current commodities (Caplan 1996; Janick et al. 1996). On the other hand, converting crop acreage into new industrial crops by decreasing production of major commodities may in the long term decrease the amount of agricultural subsidies required and will encourage the expansion of the manufacturing and industrial sector associated with the food chain.

1.3 Plants as producers of secondary metabolites

1.3.1 Definition of plant secondary metabolites

Plant metabolites are usually divided into two groups according to their species specific characters, importance to plant development and concentration of the compound dry weight (Luckner 1990). Fats and lipids, carbohydrates and proteins are referred to as primary products derived from general metabolism. These molecules have an important role in plant development and their biosynthesis is usually universal within the plant kingdom. Secondary metabolites consist of compounds the concentration of which may be only few percent or less of the dry weight. The function or importance of these compounds to plant development is not always clear, although many of them are involved in defense mechanisms or act as signaling substances in plants (Luckner 1990). Some of the most common secondary metabolites are compounds derived from glucose and acetyl-CoA known as isoprenoids; and compounds derived from aminoacids such as alkaloids, phenylpropanoids, and glucosinolates. In addition to pure compounds, secondary metabolites can be combined from precursors from different pathways of primary metabolism (Luckner 1990).

1.3.2 Importance of isoprenoids and terpenes

Isoprenoids are a large group of C_5-isoprene units containing compounds accumulated in plants, animals, and microorganisms. Isoprenoids are derived from glucose and acetyl-CoA via mevalonate pathway condensing finally into five carbon units containing compounds. Until now a vast number of different isoprenoids have been identified (Luckner 1990, Banthorpe and Charlwood 1980), the pathway of which is illustrated in chapter 4. Mevalonate pathway has been studied intensively in mammalian cells (Goldstein and Brown 1990) because cholesterol is produced by this pathway. Plant isoprenoids comprise a structurally diverse group of compounds which are sometimes divided into classes of primary and secondary isoprenoids (Chappell 1995). Terpenes contain less than five (C_5-C_{25}) (Banthorpe and Charlwood 1980) units of original carbon skeletons (Luckner 1990) whereas isoprenoids have more than five isoprene units (C_{30}->) (Banthorpe and Charlwood 1980) often with altered carbon skeletons (Luckner 1990). Examples of terpenes and isoprenoids are summarized in Table 1.

Table 1. Secondary products derived from activated isoprene (Luckner 1990)

Number of isoprene units	Group of compounds	Representatives
1	Hemiterpenes	Isoprene, 3,3-dimethylallyl alcohol, isopentenol, isoamyl alcohol, tuliposides
2	Monoterpenes	Constituents of essential oils and iridoids
3	Sesquiterpenes	Constituents of essential oils and sesquiterpene lactones, abscisic acid, juvenile hormone, sirenin
4	Diterpenes	Constituents of resins, gibberellins, phytol, vitamin A, crocetin
5	Sesterterpenes	Constituents of unsaponifiable lipid extracts, waxes
6	Triterpenes	Squalene, steroids, pentacyclic triterpenoids
7	Tetraterpenes	Carotenes, xantophylls
>8	Polyterpenes	Rubber, gutta, solanesol, spadicol, dolicols, betulaprenols, ficaprenols

1.3.2.1 Terpenes

Mono- sesqui-, and diterpenes are classified as secondary isoprenoids (Chappell 1995). They are constituents in essential oils and phytoalexins and are most often cyclic; contain relatively few simple functional groups such as hydroxyls, carbonyls, and double bonds; and commonly represent variations on a limited number of skeletal themes. Usually the boiling point range increases with size and degree of functionality (Luckner 1990). Terpenes are involved in plant-environment interactions such as defense-mechanisms, allelopathy, pollination, and attracting hormones (Luckner 1990). With the enormous number of compounds now known, to seek a role for every mono- and sesquiterpene produced in the plant kingdom seems unrealistic. As a class, however, the mono- and sesquiterpenes would appear to impart significant survival value to the plant (Croteau 1992).

1.3.2.2 Isoprenoids

The class consisting of primary isoprenoids (Chappell 1995) includes compounds essential for membrane integrity, photoprotection, orchestration of developmental programs, and anchoring essential biochemical functions to specific membrane systems (Chappell 1995). Examples of these isoprenoids include compounds incorporating into glycoprotein synthesis (dolichol), regulating cell division (GA, ABA, cytokinins) (Table 1), transporting intracellular vesicles, and active in cytoskeleton and membrane structure organization (phytosterols) (Morehead et al. 1995). All are functions fundamental for full morphogenic expression. In addition, isoprenoids play a significant role in respiration (the side chain of ubiquinone) and photosynthesis (phytol chain of chlorophylls; plastoquinone and other prenylquinones in the chloroplast; carotenoids). In addition to pure compounds, other classes of secondary metabolites may have constituents derived from mevalonate pathway (Table 2)

Table 2. Examples of secondary metabolites containing isoprenoidal constituents

Compound	Species	Reference
Anthraquinones	species in Rubiaceae and Apocynaceae	Ramos-Valdivia 1998; van der Plas et al. 1995
Cardenolides	*Digitalis lanata*	Milek et al. 1997
Indole alkaloids	*Catharanthus roseus*	van der Heijden and Verpoorte 1995; van der Heijden et al. 1994; Burnett et al. 1993
Prenylated polyphenols		Yamamoto et al. 1997; Paseshnichenko 1995
Shikonin	*Lithospermum*	Gaisser and Heide 1996
Steroidial glycoalkaloids	*Solanum* sp.	Valkonen et al. 1996
Taxol	*Taxus* sp.	David and Dayanadan 1997; Jaziri et al. 1996

2 Genetic and morphological diversity and evolution in *Tanacetum*

2.1 Taxonomy

The genus *Tanacetum* is one of the more than 100 genera in the tribe Anthemideae (Soreng and Cope 1991) which contains about 10% of the total genera and 15% of the species of Asteraceae (Heywood and Humphries 1977). Many species of at least 14 closely related genera such as *Tanacetum*, *Leucanthemum*, *Dendranthema*, *Chrysanthemum*, and *Argyranthemum* were previously classified under the genus *Chrysanthemum* (Soreng and Cope 1991; Anderson 1987). The vast complexity of *Chrysanthemum* (Soreng and Cope 1991; Anderson 1987; Heywood and Humphries 1977) lead ultimately to a division of that genus into smaller ones (Soreng and Cope 1991; Anderson 1987). Currently, *Tanacetum* consists of about 70 - 150 species depending on the classification (Abad 1995; Soreng and Cope 1991; Heywood and Humphries 1977) and is one of the largest genera in Anthemideae (Abad 1995). The reclassification based on Soreng and Cope (1991) where tansy and pyrethrum are taxonomically classified to genus *Tanacetum* is used in this thesis.

2.2 Distribution and origin

Species in *Tanacetum* are found throughout temperate regions particularly in the northern hemisphere even up to Northern Europe, Canada, Alaska, and Northern Russia (Heywood and Humphries 1977; Heywood 1976; Hultén 1968; Hultén 1950), although the center of diversity and probably also the origin for *Tanacetum* is South-West Asia and the Caucasus in the Old World (Soreng and Cope 1991; Heywood and Humphries 1977). Some endemic groups occur in Macaronesia, Himalayas, and in North America (Heywood and Humphries 1977).

Tansy, *Tanacetum vulgare* L., is rather widely distributed in Europe and many other parts of the world (Keskitalo et al. 1998a), although it was mentioned not to exist in Acores, Islas Baleares, Spitsbergen, Bear Island and Jan Mayen (Heywood 1976). Especially, in

the northern hemisphere tansy occurs as a circumpolar species (Hultén 1968). Tansy is not native to Ireland (Heywood 1976) even if it has spread on Clare Island located off the West coast of Ireland (Brodie 1991). Tansy is the only species in the genus *Tanacetum* grown naturally in Finland (Hämet-Ahti et al. 1984) where the species has spread even north of the Arctic Circle (Jalas 1991; Hämet-Ahti et al. 1984; Hultén 1968; von Schantz and Järvi 1965; Linkola and Väänänen 1940). Harbors have been common entry points for new tansy germplasm from other countries (Jutila 1996) into probably the endemic Finnish gene pool of tansy grown especially along the sea shore (Suominen and Hämet-Ahti 1993; Linkola and Väänänen 1940). As a human settlement associated species (Silkkilä and Koskinen 1990; Linkola and Väänänen 1940) tansy has spread from southern Finland where most of the harbors are located throughout the country probably linearly with the spread of inhabitation and agriculture (Keskitalo et al. 1998a; Linkola and Väänänen 1940). In addition to the role of human action, the spread of tansy occurs also by seeds dispersed by wind (Prach 1988).

Pyrethrum, *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip., originates from Albania and the area of former Yugoslavia (Heywood 1976). In the beginning of 1900's the cultivation of pyrethrum for the insecticide, pyrethrins, was introduced from Yugoslavia to Japan, Kenya, and Tanzania (Gullickson 1995; Bhat and Menary 1984b; Tuikong 1984). Later, India (Singh and Sharma 1989), Tasmania in Australia (Gullickson 1995; Bhat and Menary 1984b), China, USA, and several countries in South America (Gullickson 1995) became producers of pyrethrins. Pyrethrum has been cultivated also in several countries of Europe, namely in Austria, East-Germany, France, Hungary, Italy, Lithuania, Spain, and Russia (von Schultka et al. 1985; Heywood 1976; Ozola 1971).

2.3 Economic botany

Pyrethrum is the only species in the genus *Tanacetum* of agronomic importance, although the genus consists of several species producing similar types of bioactive metabolites (Abad et al. 1995). Tansy is aromatic and perennial species tolerant of hard Scandinavian winters (Keskitalo et al. 1998a; Jalas 1991; Linkola and Väänänen 1940). The mean height of the usually branched stem is 92 cm (40-140 cm) and the number of yellow flower heads in the corymb is 45 cm (0-179 cm) (Keskitalo et al. 1998a; Heywood 1976). Dark green leaves are pinnatipartite to pinnatisect and sparsely hairy (Heywood 1976). Tansy grows naturally in sandy or coarse soils and is modest with regard to humidity and soil nutrients (Wittig and Gödde 1985). Even on polluted soils tansy has been observed to grow over several years (Prach 1988; Terpò and Bàlint 1985). Seeds as well as vegetatively (Zàmbori-Nèmeth 1987) or micropropagated shoots (Keskitalo et al. 1998b) can be used for propagation of tansy. Flowering occurs from July to August and the flowers are mostly cross-pollinated (Lokki et al. 1973). Maturation and seed setting of a high number of small (1-2 mm), easily dispersing achenes occurs one month later (Keskitalo et al. 1998a; Prach and Wade 1992). The production is on average half a million seeds m^{-2} $year^{-1}$ but can be as much as one million seeds m^{-2} $year^{-1}$ (Prach 1988). The yield of fresh biomass and essential oils of tansy was 1.0-2.4 kg m^{-2} and 0.9-2.3 g m^{-2}, respectively, depending on the time and number of harvests (Dobos et al. 1992).

Pyrethrum is a perennial herbaceous plant that can be propagated by seeds, vegetative splits, stem cuttings rooted under mist, and tissue culture (MacDonald 1995). Many

shoots, often branched, originate from the crown of the silver-gray and pinnatipartite (Heywood 1976) leaves and grow up to 75 cm (MacDonald 1995). The vegetative period takes several months before the plant is ready for the induction of flowering. Pyrethrum is able to become reproductive either under short (8 h) or long-day (16 h) conditions. But, regardless of photoperiod, pyrethrum grown in low day and night temperature (Mohandass et al. 1986; Roest 1976) for at least six weeks induces flowering and lengthens the shape of the leaves. In contrast, the development of initiated flower heads and the vegetative development are stimulated with higher temperatures (Roest 1976). Daisy-like flower heads consist of two kinds of flowers: the disc florets in the center which are yellow and the white ray florets, which form the outer rim of the flower head (Brewer 1968). Flower yield and pyrethrins content varied in pyrethrum grown in Australia from 140 to 2400 kg ha^{-1} and from 0.06 to 1.80% DW, respectively (Bhat and Menary 1984b), whereas the pyrethrins content in Indian grown pyrethrum varied from 0.9-1.5% DW (Pandita and Sharma 1990). However, new varieties with improved pyrethrins content such as Hypy (Bhat and Menary 1984a), Hansa (Singh et al. 1988b) and a patented Arizona variety (McDaniel 1991) are now available.

2.4 Genetic variation

Genetic variation in species, e.g. individual plants in which the number and frequency of alleles in a locus varies, is an important factor for adaptation and survival of populations to different environments. Genetic variation is also the basic source for biodiversity of all species and is fundamental for life. Especially for natural populations, increased genetic variation enhances usually the overall survival of the species.

Genetic variation of plants, distinctive to environmental variation, is inherited and is a consequence of mutation, polyploidization, hybridization, gene flow, genetic drift, selection or the fertilization system where half of the genes are inherited from both of the parents (van Raamsdonk 1995). Pyrethrum is totally cross pollinating which makes the crop highly heterozygous and variable in many agronomic and other characteristics (Brewer 1968). In pyrethrum, a one-locus sporophytic self-incompatibility system has been reported (Brewer 1974), which means that self fertilization is prevented by inhibition of pollen germination of the trinucleate pollen on the stigma (Brewer 1968). The incompatibility system may also hinder crosses between related or even unrelated cultivars which has been observed in *Chrysanthemum* cut flowers (Wolff et al. 1995). Similarly, tansy was observed to be mostly cross-pollinating and thus the seed yield after self-pollination is only 1-4% of the yield after natural cross-pollination (Lokki et al. 1973). Low seed yield was observed to result also from cross-pollination of certain chemotypes (Holopainen et al. 1987a; Lokki et al. 1973) suggesting barriers of pollination between chemotypes.

Assessment of genetic variation is the first step prospecting towards domestication and improvement of a species. For this purpose, several morphological, cytogenetic, biochemical and DNA markers have been used (Bretting and Widrlechner 1995).

2.4.1 Genetic variation within the genus *Tanacetum*

Nuclear DNA content, cytogenetics of the genomic complement and molecular markers are common tools to study genetic diversity. The nuclear DNA content has been observed to vary in many *Chrysanthemum* species, even within species having the same chromosome number (Heywood and Humphries 1977). The mean nuclear DNA content of eight tansy genotypes grown less than six months *in vitro* ranged between 7.84 and 9.95 pg (mean 8.86 pg) (Keskitalo et al. 1998a) whereas the nuclear DNA content of another two genotypes grown several years *in vitro* was and 6.41 and 7.39 pg (Keskitalo et al. 1999). Nuclear DNA contents of five pyrethrum clones ranged between 13.16 and 14.76 pg (Keskitalo et al. 1999).

The basic chromosome number of tansy and pyrethrum as well as other species in the tribe Anthemideae is 2n=2x=18 (MacDonald 1995; Heywood and Humphries 1977; Virrankoski and Sorsa 1968). However, triploid (2n=3x=27) (MacDonald 1995; Ottaro 1977) and tetraploid (2n=4x=36) pyrethrum clones have also been identified (MacDonald 1995). The chromosome number of tansy has been reported to be stable (Heywood and Humphries 1977) even if cytomictic disturbances in the pollen mother cells (PMCs) have been observed (Virrankoski and Sorsa 1968) which could explain the wide variation in the nuclear DNA content (Keskitalo et al. 1998a).

All of the 20 tansy genotypes studied in this thesis could be distinguished by RAPD analyses (Keskitalo et al. 1998a). The reason for the detected, relatively high polymorphism in this species (mean genetic distance 0.294) is not clear, but it may be partially due to the high frequency of cross pollination (Lokki et al. 1973). In the present thesis multivariate analysis of RAPD patterns clustered the 20 tansy genotypes collected in Finland into two groups which divided further into smaller groups. Geographically, genotypes originating close to each other were also genetically similar. The two major groups formed as a result of multivariate analyses were geographically originated from south and north of the latitude of 60°30’N. The two clusters differed in flower number, length of corymb, number of nodes and date of flowering (Keskitalo et al. 1998a). Tansy, pyrethrum, and the protoplast-fusion derived calli were also distinguished by RAPD-PCR (Keskitalo et al.1999).

Pyrethrum has been observed to vary genetically by many authors (Singh and Singh 1996; Singh et al. 1988b; Singh et al. 1987b; Parlevliet 1975), although studies based on DNA or protein markers are not available.

2.4.2 Genetic variation in species related to *Tanacetum*

Tansy, pyrethrum, and most of the other species in the genus *Tanacetum* were previously classified to belong to the genus *Chrysanthemum*, and still a few authors hesitate their taxonomical status (Heywood Humphries 1977). Thus, also species belonging to the genus *Chrysanthemum* are frequently referred to in this thesis. Several techniques of DNA analysis such as RAPDs, inter-SSR PCR (simple sequence repeat polymerase chain reaction), hybridization based on DNA fingerprinting as well as RFLPs (restriction fragment length polymorphism) distinguished unrelated *Chrysanthemums* used as cut flowers (Wolff et al. 1995). In contrast, but not surprisingly, different vegetatively

propagated mutations differing in flower color and derived from one cultivar had very little intraspecific genetic polymorphism (Wolff et al. 1995). There was no correlation between molecular and morphological characters of chrysanthemum flowers (Scott et al. 1996). Also, no relationship between the ploidy level of *Dendranthema* species and the number of RAPD fragments generated was observed (Wolf and Peters-van Rijn 1993). The genetic identity based on isozyme loci within the genus *Chrysanthemum*, namely between *C. coronarium* and *C. segetum* was 0.84 (Francisco-Ortega et al. 1995).

Genetic diversity between *Tanacetum* and related genera has received limited research attention. Based on isozyme analyses the genus *Argyranthemum* (Asteraceae) was observed to be equally divergent from three other related genera grown in the Mediterranean, namely *Chrysanthemum*, *Heteranthemis* and *Ismelia* suggesting its role as an ancestor for these genera. In contrast, genetic identity between *Chrysanthemum* and *Ismelia* was high (0.93) and it has been suggested that these genera should be treated as one genus (Francisco-Ortega et al. 1995).

2.5 Morphological variation among tansy and pyrethrum

In prospecting a new species towards domestication, one of the most important goals of breeding is to achieve uniformity in morphological characters. However, before breeding programs can be initiated, it is important to explore the inherent variation in morphological characters. Among wild species morphological variation is usually very wide indicating adaptability to different environmental conditions.

Variation in flower heads in a corymb, the shoot height, branching, the length and width of leaves, dissection of leaves, hairiness, and the color of flower heads have been observed also in tansy (Keskitalo et al. 1998a; Németh et al. 1994; Hämet-Ahti et al. 1984; Heywood 1976). Morphological subvarietes of tansy with differentially dissected (*Tanacetum vulgare* forma *týpicum*, and forma *tenuiséctum*) (Heywood 1976; Forsén 1975; von Schantz and Järvi 1965) or curly leaves (*Tanacetum vulgare* forma *crispum*) (Hämet-Ahti et al. 1984; Forsén 1975; von Schantz and Järvi 1965) have been reported. Their genetical status within *T. vulgare* is, however, unclear.

Although, pyrethrum has been cultivated for decades there is still quite an extent of variation in morphological characters. Several studies show that morphological variation in flower size, flower yield, flower number, flower weight, bush diameter, plant height and lodging within pyrethrum is very wide (Singh and Sharma 1989; Singh et al. 1988b; Pandita and Bhat 1987; Singh et al. 1987a; Singh et al. 1987b; Bhat and Menary 1986; Bhat et al. 1985; Parlevliet and Contant 1970; Parlevliet 1969). The ploidy level was observed to cause alteration in morphology, thus the triploid pyrethrums had larger and heavier flowers, the stem of the plants was longer, and they had fewer but longer stomata compared to diploid plants (Ottaro et al. 1977). The observed wide morphological variation may be a consequence of the production system of pyrethrum. For decades, small scale farmers have harvested flowering pyrethrum by hand and there has been no need for varieties with more uniform flowering. However, since 1980's many developed countries such as USA and Australia have been breeding new uniform varieties suitable for mechanized plant production (McDaniel 1991; Bhat and Menary 1984a).

Several morphological characters of pyrethrum have shown parent-offspring heritability and thus are suitable as morphological markers (Bretting and Widrlechner 1995) in breeding programs. Heritable characters include: number of flowers per plant (Singh et al. 1988a), flower weight (Singh et al. 1988a), flower size (Parlevliet and Contant 1970), flower yield (Parlevliet and Contant 1970), pyrethrins content (Singh et al. 1988a; Parlevliet and Contant 1970), pyrethrins yield (Singh et al. 1988a), and resistance to lodging (Parlevliet and Contant 1970).

2.6 Interaction between characters

Biodiversity, i.e. variation in morphology as well as genetic, and chemical composition, is a value in itself. However, a question arises whether there is a correlation between these traits and whether it would be possible to explain variation in a particular character?

The link between genetic and chemical variation has interested researches. In this thesis research, we observed 41% analogy between similarity matrices of RAPD-PCR patterns and variation of 55 volatile compounds analyzed by the same statistical method (Keskitalo et al., unpublished; Keskitalo et al. 1998a). In the previous study, it was observed that thujone and camphor lines and also two different thujone lines of tansy were genetically distinct, and that the synthesis of thujone is controlled by dominant alleles whereas sabinene by recessive alleles (Holopainen et al. 1987a; Lokki et al. 1973).

Morphological characters such as plant height, number of branches, yield of flowers and yield of shoots was observed to vary according to the main compounds in tansy essential oil (Németh et al. 1994). Similarly, in this thesis we detected variation in morphology between tansy chemotypes (Keskitalo et al., unpublished). Pyrethrins yield in pyrethrum was correlated to flower yield, pyrethrins content, number of flowers per plant, dry weight of flowers (Singh et al. 1987b; Bhat and Menary 1986), and especially to flower diameter (Pandita and Bhat 1987; Singh et al. 1987b). In addition to morphology, the concentration of isoprenoids in tansy (Németh et al. 1994; Holopainen 1989; von Rudloff and Underhill 1965; von Schantz and Järvi 1965) and pyrethrum (Bhat and Menary 1984c) depend on the developmental stage being the highest in tansy at the onset of flowering (von Schantz et al. 1966) and in pyrethrum when three-quarters of the disc florets have opened (Bhat and Menary 1984c).

Tansy genotypes of different geographical origin have been observed to contain wide variation of monoterpenes (Keskitalo et al., unpublished; Collin et al. 1993; Nesmélyi et al. 1992; Hendriks et al. 1990; De Pooter et al. 1989; Holopainen 1989; Gallino 1988; Héthelyi et al. 1981; Nano et al. 1979; Forsén 1975; Sorsa et al. 1968) but the reason for this is still under investigation. When grouped according to the main terpenes detected from the 20 tansy genotypes studied in the present thesis we found eight chemical clusters. Genotypes originating geographically from neighboring areas usually clustered to the same chemical group. Latitude-effect was observed in genotypes containing high concentrations of camphor, as these genotypes originated usually from Central Finland whereas those without or with a low concentration of camphor originated from Southern-Finland (Keskitalo et al., unpublished; Keskitalo et al. 1998a). This observation is supported by an earlier study, where genotypes containing camphor were frequently found from Northern-

Finland (Sorsa et al. 1968). In addition to the latitude, also altitude has been observed to favor isoprenoid biosynthesis such as pyrethrins in pyrethrum (Singh et al. 1988b).

In the present study of tansy, variation in morphology as well as genetic and chemical composition was found to correlate in some cases. This was especially the case when the results of the different groups were compared. The two major groups formed based on the multivariate analyses of RAPD-PCR patterns could be distinguished also by selected morphological characters and date of flowering. Also, the variation in volatile compound composition correlated with the variation of RAPD-PCR patterns, although the correlation was not strong. In addition to the links between morphology and genetic and chemical composition, all of these traits were associated with the geographical origin of the genotypes (Keskitalo et al., unpublished; Keskitalo et al. 1998a).

3 Diversity and importance of isoprenoids in *Tanacetum*

3.1 Importance of isoprenoids

3.1.1 Previous uses of tansy

A rich and imaginative history of the application for medicine, preservation, insect control and as a spice and food ingredient has followed tansy from Medieval to modern times. The wide scale of applications from toxic to nutritive substances (Table 3 and 4) indicate a variation in essential oils and/or in other compounds with bioactivity.

Table 3. Applications of tansy (Duke 1985; Grieve 1984; Hussey 1974; Millspaugh 1974)

Application	
For foodstuff	Cakes, cheese, herbal remedies, omelets, pudding, salads, spicing, tea
Medicinal uses	abortion, amenorrhea, bruises, burns, cholecystosis, chorea, cold, dropsy, dysmenorrhea, epilepsy, eyes, fever, freckles, gout, hepatosis, hydrofobia, hysteria, inflammation, kidney problems, labial abscess, leucorrhea, nerves, palpitation, paralysis, rheumatism, sciatica, sores, spasms, sprains, stabismus stomachache, sunburn, swellings, toothaches, tuberculosis, tumors, worms
Other uses	Cosmetic, dyes, embalming, insect control, ointments, perfumeries

The name, *tanacetum*, is derived from Greek (*a)thanatos*, (*thanatos* = death), in reference to tansy's medicinal qualities, everlasting scent, and preservative uses as well as its use for embalming the dead (Mitich 1992). Flowers, leaves or distillate of tansy were shown to repel insets. Thus, especially during Easter, the time when fish was frequently included in the diet, the food was supplemented with tansy to cure for intestinal worms and to counteract illnesses due to the cold and humid weather. Eventually, tansy become a desirable constituent of puddings and omelettes and had an honorable place in culinary writings. Even cakes prepared from tansy, which were called tansies, are mentioned in the history (Mitich 1992; Grieve 1984). Roots of tansy, especially if preserved with honey and sugar were used for gout, and the flowers steeped in vodka for stomach and duodenal

ulcers (Grieve 1984; Hussey 1974; Millspaugh 1974). Tansy has been used externally for skin diseases and to lighten face from freckles and as an ingredient in fragrances and dyes (Mitich 1992; Grieve 1984). Sometimes tansy was cultivated as an ornamental plant in some areas (Heywood 1976).

Table 4. Specific medicinal and insecticidal activity of tansy (Duke 1985; Grieve 1984; Hussey 1974; Millspaugh 1974)

Specific activity	abortifacient, antibiotic, anthelmintic, antioxidant, antiseptic, ascaricide, bactericide, cordial, diaphoretic, emmenagogue, narcotic, nervine, pediculicide, poison, pulicide, sedative, stimulant, stomachic, sudorific, tonic, vermifuge, vulnerary

3.1.2 Modern applications of bioactivity in tansy

3.1.2.1 Medicinal activity

Recently published studies show that extracts of tansy have anti-inflammatory activity against human polymorphonuclear leukocytes (Brown et al. 1997), mouse-ear edema (Schinella et al. 1998), and carrageenan-induced edema in rats (Mordujovich-Buschiazzo et al. 1996). It is assumed that sesquiterpene lactone, parthenolide, which was found in considerable amounts in tansy (Schinella et al. 1998) is responsible for the activity (Schinella et al. 1998; Brown et al. 1997). Tansy also belongs to a group of plant species known as cancer remedy in folk-medicine. In fact, tansy extract was shown to have such activity in the NIH (US National Institute of Health) cancer program (Duke 1985). More specifically, the α-methylene-γ-lactone ring is known to have anticancerogenic activity (Stefanoviç et al. 1985). The phytochemical and pharmacological utility of tansy and other species in the genus *Tanacetum* was recently reviewed (Abad et al. 1995).

3.1.2.2 Antimicrobial activity

Essential oil and extracts have shown antibacterial and -fungicidal activity, the effect which seems to depend on the composition of the oil. Thujone-type oil from tansy showed highest and camphor, sabinene, and umbellulone-type oils slightly lower activity at a concentration of 5-20% against Gram-positive bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* (Holopainen and Kauppinen 1989). Essential oil distilled from tansy genotype containing *trans*-chrysanthenyl acetate (75%) and *trans*-chrysanthenol (5-10%) inhibited the growth of fungi and most of the Gram-positive bacteria (Neszmélyi et al. 1992). The toxic activity of essential oils from different chemotypes of tansy grown in Hungary varied when tested against 40 species of microorganisms. The oils inhibited the growth of Gram-positive bacteria such as *Staphylococcus* and *Streptococcus*, yeast, fungi and some Gram-negative bacteria (Héthelyi et al. 1991; Héthelyi et al. 1989). Sesquiterpene lactones of tansy had bactericidal activity against *Staphylococcus aureus* whereas the activity was slightly lower against *Salmonella* sp. It was assumed that the α,β-unsaturated-γ-lactone ring played a decisive role in promoting the biological activities (Stefanoviç et al. 1988).

3.1.2.3 Insecticidal activity

A distinct advantage of plant-derived chemicals used for pest control in agriculture is that they often degrade fast and thus do not leach to the groundwater. Moreover, the complexity of natural extracts effectively hinders the development of resistance in the pests. The insecticidal activity of tansy has been reported to be due to the repellent action (Nottingham et a. 1991; De Pooter et al. 1989; Suomi et al. 1986; Schearer 1984), prevention of oviposition, contact toxication (Luik and Ploomi 1995), water loss of pupaes (Harak 1994), deterring effect (Suomi et al. 1986), reduced feeding, increased pupation period, and decreased weight of pupae (Hough-Goldstein and Hahn 1992) on several pests (Table 5).

Table 5. Insecticidal activity of tansy

Insect	Symptoms	Formula	Reference
Aedes aegyptii	repellent	essential oil	De Pooter et al. 1995
Brevicoryne brassicae	repellent	odors of tansy	Nottingham et al. 1991
Cydia pomonella	repellent	chloroform extracts	Suomi et al. 1986
Leptinotarsa decemlineata	repellent	essential oil	Panasiuk 1984; Schearer 1984
Leptinotarsa decemlineata	reduced the number	interplanted with potato	Matthews et al. 1983
Myzus persicae	reduced the number	interplanted with bell pepper	Matthews et al. 1983
Pieris brassicae	inhibited oviposition	water extract	Luik and Ploomi 1995
Pieris brassicae	water loss of pupas	water extract	Harak 1994
Pieris rapae	reduced feeding, reduced eggs laid, lengthened the pupation period, decreased weight of pupae	water extract	Hough-Golstein and Hahn 1992
Plutella xylostella	reduced eggs laid	water extract	Hough-Goldstein and Hahn 1992

The steam distillate of fresh leaves and flowers of tansy was strongly repellent to Colorado potato beetles (*Leptinotarsa decemlineata*). The major components of that particular tansy oil were camphor (30%) and umbellulone (20%). A commercial oil of tansy containing bornyl acetate (74%) as the main component was also highly repellent to the beetles. As single compounds, also 1,8-cineole, ρ-cymene, γ-terpinene and camphor repelled the beetles (Schearer 1984). In a detailed study on the repelling action, Colorado potato beetles exhibited avoidance to tansy oil, volatiles from intact tansy plants, a hydrocarbon fraction of tansy and seven of the known 13 components of tansy oil tested. Deterred components of tansy oil were α- and γ-terpinene, ρ-cymene, α- and β-thujone,

dihydrocarvone, and carvone whereas α-pinene acted as an attractant (Panasiuk 1984). The LaPine Scientific Co. (Norwood, New Jersey, USA) was mentioned to have a product, "Oil of Tansy", consisting of essential oil from tansy having insecticidal properties (Schearer 1984).

3.1.2.4 Attractant properties

In addition to the insecticidal properties, plant extracts and essential oils may also have attractant properties to insects. The function of the oil depends on the composition (Panasiuk 1984; Gabel et al. 1992) and the concentration of compounds present in the oil. Insects may react differently to even the same plant extracts (Gabel et al. 1992).

Tansy attracted European grapevine moth females (*Lobesia botrana*) to harvest nectar or pollen from its flowers, although the moth never ovipositioned or the larval development occurred on tansy (Gabel 1992; Gabel et al. 1992). According to the authors (Gabel et al. 1992), the odor from tansy could be used to interfere with the orientation of females, lure females outside the vineyards, monitor the population dynamics, or directly control the population by mass trapping. It seems possible that European grapevine moth females seek for tansy-specific chemicals of which ρ-cymene, d-limonene, α-thujene, α-thujone, β-thujone, thujyl alcohol, terpinen-4-ol, verbenol and piperitone caused an elicited response (Gabel et al. 1992).

3.1.2.5 Other uses

The rare bicyclic monoterpene ketone, chrysanthenone possesses a powerful deep green, slightly oily-floral aroma suitable for aromatic industry (Lawrence 1992). Also other compounds present in tansy may find industrial uses as flavoring agents (Appendino et al. 1988). Tansy was observed to be a potential dye plant giving bright yellow color on wool (Anon. 1997).

3.1.3 Applications of bioactivity in pyrethrum

Pyrethrum is the only plant species the metabolites of which are currently commercially exploited in insecticides. Pyrethrum produces insectisidal compounds collectively termed as pyrethrins which are a combination of six esters: cinerin I, jasmolin I, pyrethrin I, cinerin II, jasmolin II, and pyrethrin II (Head 1969). Pyrethrins cause a rapid knock down and paralyzing effect on Arthropod nerves (Camougis 1973) but have a low mammalian toxicity (Griffin 1973). Thus, pyrethrins are widely used as environmentally benign insecticides in agriculture, horticulture (Silcox and Roth 1995), homes (Kennedy and Hamilton 1995), stored products (Silcox and Roth 1995), and for pest control in animals (Gerberg 1995). Sesquiterpene lactones in pyrethrum flowers have also inhibiting activity on seed germination (Sashida et al. 1983). The increased demand of natural pyrethrins has facilitated Australia to establish a highly mechanized production of natural insecticides (Johnson 1989). The wholesale price for technical grade pyrethrum extracts was reported to be \$413 kg^{-1} on a 100% basis. In 1992 the world market for the natural pyrethrum insecticide was estimated to be \$400 million annually. Conventional production of

pyrethrins is still well below the global market demand (Jovetiç and de Gooijer 1995). Absence of strong resistance to pyrethrins in insects and the pressure to replace harmful synthetic insecticides has led to an increased demand on natural pyrethrins (Johnson 1989).

3.2 Variation of isoprenoids

Mono- and sesquiterpenes in addition to flavonoids are the most important secondary compounds detected in *Tanacetum* sp. belonging to Anthemideae, the tribe which includes many aromatic plants of medicinal and pesticidal properties in the family Asteraceae. Due to their chemosystematic value these chemicals have been the interest of many researchers for decades (Greger 1977).

3.2.1 Tansy

3.2.1.1 Volatile isoprenoids

More than 100 volatile terpenes have been isolated and identified from tansy (see Appendix I). The essential oil content of tansy flowers is about twice as much as in the leaves (Dobos et al. 1992). During sprouting the essential oil content in the leaves can be as high as 0.9% FW, and the content decreases towards flowering depending on the chemotype (Németh et al. 1994). The essential oil content in the harvested tansy leaves and flowers varied between 0.1-0.3% and 0.1-0.4%, respectively (Collin et al. 1993; Neszmélyi et al. 1992), whereas the dry leaves and flowers contained up to 0.5-0.9% and 0.7-1.8% of essential oil, respectively (Hendriks et al. 1990). In contrast to leaves, in flowers the essential oil composition is the highest in the beginning of flowering (Németh et al. 1994; Holopainen 1989; von Schantz et al. 1966; Rudloff and Underhill 1965). Only part of the volatiles exist as major compounds whereas most of them are detected only as traces. Many of the volatiles detected from tansy are bioactive as described in chapter 2.

3.2.1.2 Non-volatile isoprenoids

Several partially volatile or non-volatile isoprenoids have been detected from tansy. Petals of tansy contained up to 0.06% monoterpenes of which half were as ß-glucosides of α-thujone, ß-thujone (neoisothujol and isothujol), α-terpineol or terpinen-4-ol (Banthorpe and Mann 1971). In addition, at least 44 sesquiterpenoids with different molecular skeletons such as eudesmanolides, germacranolides, and guaianolides as well as few triterpenoids and sterols have been detected from tansy (see Appendix II). Sesquiterpenes and sesquiterpene lactones are characteristic of the tribe Anthemideae and have been used for chemosystematic purposes (Greger 1977). Although also other species in the genus *Tanacetum* have been reported to contain several types of sesquiterpenes (Abad et al. 1995; Greger 1977), chemical composition of tansy is the most intensively studied so far. Especially, sesquiterpene lactones have shown antimicrobial (Stefanoviç et al. 1988), pesticidal (Greger 1977), and medicinal (Abad et al. 1995) bioactivity.

3.2.1.3 Other non-volatiles and non-isoprenoid compounds

Glucosides (Chandra et al. 1987a) and four different water-soluble polysaccharides were identified from the inflorescences of tansy. The main component was D-galacturonic or uronic acid conjugated with neutral monosaccharides such as galactose, glucose, arabinose, xylose, and rhamnose (Yakovlev and Sysoeva 1982). Also, fatty-acids (Chandra et al. 1987a); at least 15 flavonoids (Schinella et al. 1998; Ivancheva and Behar 1995; Wollenweber et al. 1989; Chandra et al. 1987a; Stefanoviç et al. 1985; Ognyanov and Todorova 1983; Appendino et al. 1982b; Adikhodzhaeva et al. 1977; Wagner et al. 1972; Michaluk and Sendra 1964); and tanning substances such as catechin and leucoanthocyanins (Shalamova and Sysoeva 1985) have been detected. During the flowering the roots of tansy contained the highest concentration of polyacetylenic (I) and thiophenic (II) compounds namely ponticaepoxide (I), artemisiaketone(tetradeca-triin-2,4,6-en-8-on-12)(I), alkinolacetat (I), dehydromatricaria ester (I), and alkinylthiophen (II) (von Schulte et al. 1966).

Many flavonoids in other plant species have recently shown to posses health promoting or medicinal activity. Thus, wide use of tansy in homeopathic medicine can be explained by the flavonoids detected recently from the flower heads of tansy (Schinella et al. 1998; Ivancheva and Behar 1995). The sulfur containing compounds such as alkinylthiophenen resembles the thiophenes detected from *Tagetes* possessing high nematocidal activity (Breteler and Ketel 1993; Kyo et al. 1990).

3.2.2 Pyrethrum

3.2.2.1 Volatile isoprenoids

Only a few studies are available addressing volatiles, i.e. monoterpenes of pyrethrum flowers. Steam distillate of Dalmatian and English pyrethrum flowers contained a hydrocarbon $C_{19}H_{40}$ which was not found from Kenyan flowers. The oils had a high acid value and their odor resembled furfural but its presence could not be confirmed (Merrit and West 1938 referred by Head 1969). The composition of the steam distillate of pyrethrum flowers was observed to be extremely complex (Head 1969). However, volatile oil material of factory origin was observed to contain naphthalene although it could not be detect from a steam distillate (Head 1969). Precursors of pyrethrins which may be volatile such as chrysanthemic acid (Barthomeuf et al.1996; Zito 1994; Zito et al. 1991; Zito and Tio 1990; Kueh et al. 1985), chrysanthemic dicarboxylic acid (pyrethric acid) (Barthomeuf et al. 1996; Zito 1994; Zito and Tio 1990), and chrysanthemyl alcohol (Zito et al. 1991; Zito and Tio 1990) have been identified from explant derived callus of pyrethrum. In addition to these precursors, leaves of *in vitro* grown plantlets contained also geraniol (Zito and Tio 1990).

3.2.2.2 Pyrethrins and other less volatile isoprenoids

Currently, the best pyrethrum varieties contain up to 3% (DW) of pyrethrins (Ian Folder 1996, personal communication). The crude extracts of pyrethrum flowers contain about

30-35% of the active constituents, pyrethrins, which are a mixture of six esters, namely cinerin I, jasmolin I, pyrethrin I; and cinerin II, jasmolin II, pyrethrin II (Table 6).

Table 6. Composition of non-volatile isoprenoids in dry flowers of pyrethrum

Compounds	Concentration % 1	2	Reference
Pyrethrins:	total 2.0	30-35	Head 1969; Head 1966
Pyrethrin I;	0.92	14.8	Head 1969: Head 1966
Cinerin I	0.18	2.2	Head 1969; Head 1966
Jasmolin I	0.09	1.2	Head 1969; Head 1966
Pyrethrin I	0.65	11.4	Head 1969; Head 1966
Pyrethrin II	1.08	15.2	Head 1969; Head 1966
Cinerin II	0.26	3.5	Head 1969: Head 1966
Jasmolin II	0.10	1.2	Head 1969; Head 1966
Pyrethrin II	0.72	10.5	Head 1969; Head 1966
Sesquiterpene lactones:			
Cadinene	-[3]	-[3]	Maciver 1995
ß-Farnesene	-	-	Maciver 1995
Germacranolides;	-	-	
Chrysanin	-	-	Doskotch et al. 1971
Chrysanolide	-	-	Doskotch et al. 1971
Pyrethrosin (chrysathin)	-	-	Head 1969
Tatridin A	-	-	Sashida et al. 1983
Tatridin B	-	-	Sashida et al. 1983
Dihydro-β-cyclopyrethrosin	-	-	Sashida et al. 1983; Doskotch et al. 1971
11,13-dihydrotatridin A	-	-	Sashida et al. 1983
11,13-dihydrotatridin B	-	-	Sashida et al. 1983
6-O-β-D-glucosyl-11,13-dihydrotatridin B	-	-	Sashida et al. 1983
Muurolene	-	-	Maciver 1995
Pyrethrol	-	-	Head 1969
Sterols:			
β-Amyrin	-	-	Head 1969
Taraxasterol (Pyrethrol)	-	5.0	Doskotch et al. 1971; Head 1969
Carotenoids:		total 0.82	Head 1969
α- and β-Carotene	-	-	Head 1969
Neurosporene like carotenoids	-	-	Head 1969
Lutein diesters	-	-	Head 1969
Flavoxanthin like carotenoids	-	-	Head 1969
Lutein monoesters	-	-	Head 1969
Lutein	-	-	Head 1969
Chlorophylls:	-	0.1	Head 1969

1 Concentration in the dry flowers, %
2 Concentration in crude extracts of pyrethrum flowers, %
[3] Concentration not detected

Pyrethrins I (cinerin I, jasmolin I, pyrethrin I) are derived from an irregular monoterpene, chrysanthemic (monocarboxylic)acid, whereas the latter, pyrethrins II (cinerin II, jasmolin II, pyrethrin II) are derived from chrysanthemic dicarboxylic acid (pyrethric acid) (Head 1969; Head 1968). The insecticidal activity increases with the increased ratio of pyrethrin

I/pyrethrin II which varies between 0.9 - 1.3 depending on the origin of the pyrethrins (Maciver 1995). Most of the pyrethrins are accumulated in achenes (93.7 %), but there are also minor quantities in disc florets (2.0 %), ray florets (1.7 %), and receptacles (2.6 %) (Head 1966). Recently, the first commercially available pyrethrin standard including all of the six pyrethrins compounds became available (Moorman and Nguyen 1997). In addition to pyrethrins (30-35%), the crude extracts of pyrethrum also contains small amounts of other isoprenoid compounds such as carotenoids (0.82%), chlorophylls (0.1%), and taraxasterol (5.0%) (Table 6).

3.2.2.3 Other non-volatiles and non-isoprenoid compounds

The remaining 65-70 % of the crude extracts in the non-insecticidal part of pyrethrum, consists of non-hydroxy fatty acids (20 %), hydroxy-fatty acids (20 %), alkanes (4%), and ceryl alcohol (concentration unknown) (Head 1969; Head 1968). Pyrethrum flower was observed to contain also alkaloids (Glennie and Harborne 1972); alkanes (Head 1969); dark brown gum (Doskotch et al. 1971); flavonoids (Sashida et al. 1983; Glennie and Harborne 1972); methylated phenol (Glennie and Harborne 1972); pyrethrol (Head 1969); resin oil (Doskotch et al. 1971); and sesamin (Doskotch et al. 1971).

3.3 Biosynthesis of isoprenoids

The overall importance of primary and secondary isoprenoids for plant development was discussed in chapter 1. Until now two distinct pathways have been detected from eucaryotic cells leading to the formation of isopentenyl pyrophosphate (IPP), the basic units of all isoprenoids.

3.3.1 Synthesis of IPP via mevalonate pathway

Mevalonate pathway (Fig. 1), also called the classical pathway for isoprenoids, has received particular attention in mammalian systems due to its critical role of mevalonate formation in cholesterol biosynthesis (Chin et al. 1982). Because cell growth is dependent on the accumulation of HMG-CoA (3-hydroxy-3-methyl-glutaryl CoA), the regulation of HMGR-CoA (3-hydroxy-3-methyl-glutaryl CoA reductase), which converts HMG-CoA to mevalonate, has been studied also for its possible role as an inhibitor for the growth of cancerous cells (Goldstein and Brown 1990). The HMGR-CoA was isolated for the first time from a cDNA library prepared from ovary cells of Chinese hamster (Chin et al. 1982). Later, the gene family coding for HMGR-CoA (*hmg*-family) was observed to consist of three genes (*hmg*1-3) in *Camptotheca acuminata* (Maldonano-Mendoza et al. 1997); four (*hmg*1-4) in tomato (Park et al. 1992); two (*hmg*1,2) in *Arabidopsis* (Enjuto et al. 1994); three (*hmg*1-3) *in Hevea brasiliensis* (Chye et al. 1992); and four (*hmg*1-4) in wheat (Aoyagi et al. 1993). In potato there are three genes (*hmg*1-3) in the family encoding HMGR-CoA (Choi et al. 1992), and seven members (*hmg*1.2-1.8) in the subfamily of *hmg*1 (Bhattacharyya et al. 1995). The basic carbon skeleton of C_5-units in isoprenes is derived from glucose, which passes through glycolysis and yields acetyl CoA (Paré and Tumlinson 1997) (Fig. 1).

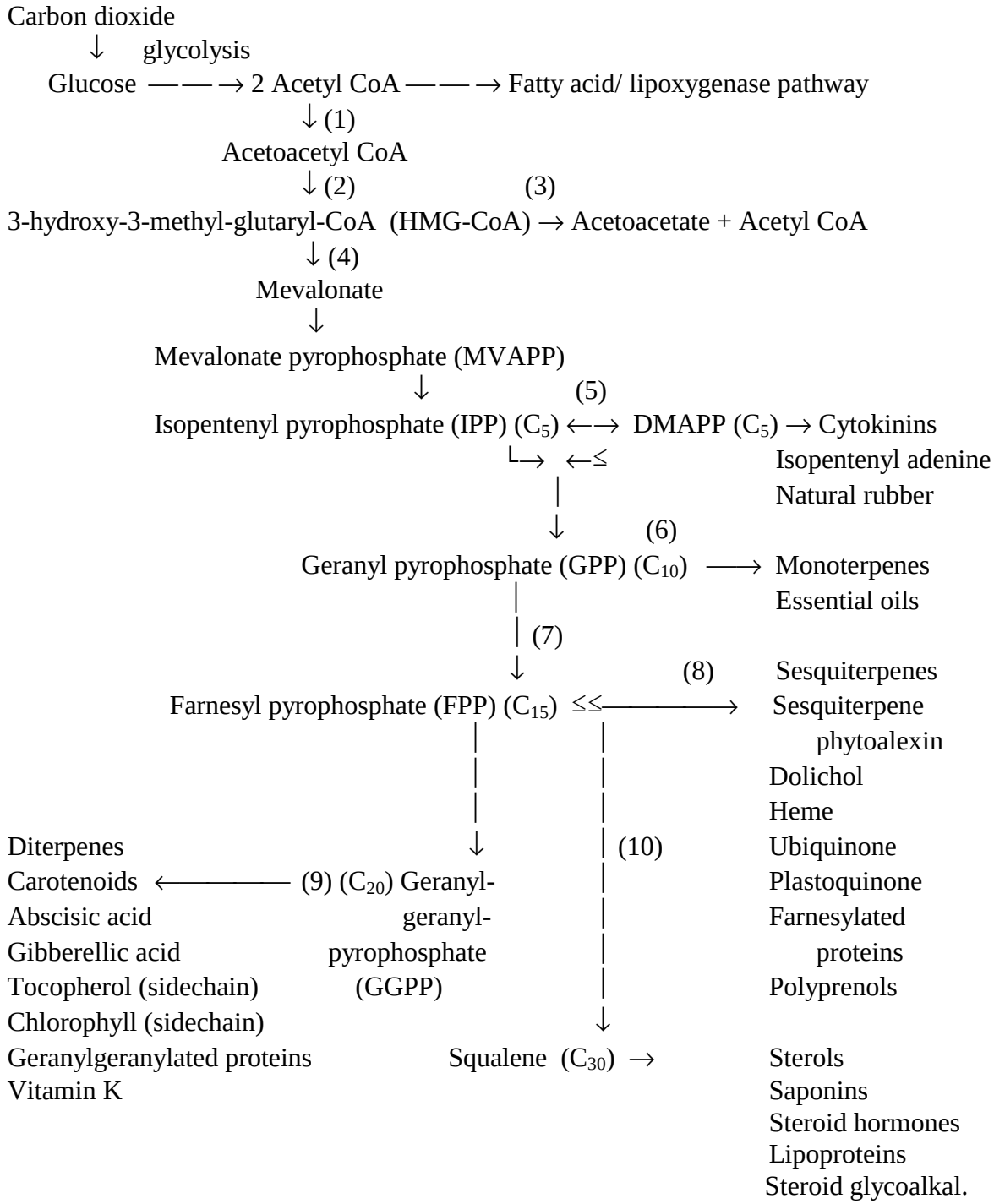

Figure 1. Simplified isoprenoid biosynthesis and the enzymes involved via mevalonate pathway in plants. DMAPP, dimethylallyl pyrophosphate. Enzymes: (1) Acetoacetyl-CoA thiolase; (2) HMGS-CoA synthase (hydroxy-methyl-glutaryl CoA synthase); (3) HMGL-CoA lyase (hydroxy-methyl-glutaryl CoA lyase); (4) HMGR-CoA reductase (hydroxy-methyl-glutaryl CoA reductase); (5) IPP isomerase; (6) monoterpene synthases and monoterpene cyclase; (7) prenyltransferase; (8) sesquiterpene synthases and sesquiterpene cyclase; (9) diterpene synthase and cyclase; (10) squalene synthase (Modified from Paré and Tumlinson 1997; Chappell 1995; Weissenborn et al. 1995; van der Heijden et al. 1994; van Kush 1994).

In the reaction which most obviously occurs in the cytoplasm, two acetyl CoA and one condensed acetoacetyl CoA form HMG-CoA. The next step where HMGR-CoA catalyzes the NADPH-dependent reaction of HMG-CoA into mevalonate, is a rate limiting step (Schaller et al. 1995; Suvachittanont and Wititsuwannakul 1995) or at least provides some control (Korth et al. 1997; Chappel et al. 1995) for the synthesis of isoprenoids and other compounds containing isoprenoid constituents (Maldonado-Mendoza et al. 1997). Mevalonate is decarboxylated and phosphorylated to yield five-carbon isopentenyl pyrophosphate (IPP), which serves as the building block for monoterpenes and other isoprenoids (Fig.1).

3.3.2 Novel synthesis of IPP via deoxyxylulose pathway

Recently, an alternative biochemical pathway for isopentenyl pyrophosphate was detected in plastids such as chloroplasts and mithochondria (Bouvier et al. 1998; Arigoni et al. 1997; Lichtenthaler et al. 1997a). Two cDNAs that encode transketolases of the novel pathway were characterized from *Capsicum annuum* plastids. CapTKT1 is involved in pentose phosphate and glycolytic cycle integration whereas CapTKT2 catalyzes the formation of 1-deoxy-xylulose-5-phosphate (Bouvier et al. 1998), a distant precursor of IPP (Bouvier et al. 1998; Lichtenthaler et al. 1997a), from glucose-derived glyceraldehyde-3-phosphate (GAP) and pyryvate (Bouvier et al. 1998). It was also demonstrated that the C_3 subunit derived from GAP and the C_2 subunit derived from pyruvate condensed via 1-deoxy-D-xylulose-5-phosphate (Schwender et al. 1996) into 2-C-methyl-D-erythiol-4-phosphate (Duvold et al. 1997). The methylerythiol should be considered as an hemiterpene and as a possible biosynthetic precursor for IPP (Duvold et al. 1997) (Fig. 2).

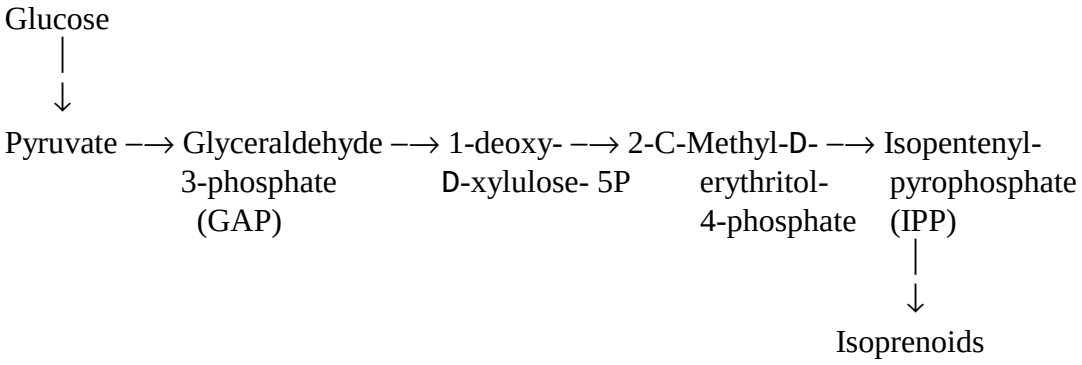

Figure 2. The novel IPP-biosynthesis in plants which most obviously occurs in the plastids (Modified from Duvold et al. 1997; Lichtenthaler et al. 1997a; Lichtenthaler et al. 1997b).

3.3.3 Further synthesis from IPP to downstream

Prenyltransverases catalyze the polymerization reaction between IPP and its isomer 3,3-dimethylallyl diphosphate (DMAPP) to form geranyl pyrophosphate (GPP) containing ten carbon atoms. Further reactions with the formed GPP and a new unit of IPP leads to farnesyl pyrophosphate (FPP) containing 15 carbon atoms. More or less volatile and lipophilic mono-, sesqui- and diterpenes of essential oils are derived from the acyclic GPP (mono- and diterpenes) and FPP (sesquiterpenes) catalyzed by mono-, sesqui- or diterpene cyclases, respectively (Chappell 1995). The prenyltransferases catalyze carbon-carbon bond formation between substrate molecules, whereas the cyclases catalyze an intramolecular cyclization. The cyclases are very substrate specific, although they can utilize the same substrate to produce totally different reaction products (Chappell 1995). Further transformations include oxidation and/or hydroxylation of cyclized mono- and sesquiterpenoids which are generally responsible for imparting the biological function of these compounds (Croteau 1992). The polymerization of IPP with GPP or FPP can continue resulting in isoprenoids which may contain thousands of C_5-units such as in rubber (Kush 1994).

It is assumed that chloroplast bound isoprenoids (ß-carotene, lutein, prenyl chains of chlorophylls and plastoquinone-9) may be formed via the novel glyceraldehyde-3-pyrophosphate (GAP)/pyruvate pathway (Bouvier et al. 1998; Lichtenthaler et al. 1997a and 1997b) whereas the cytoplasmic sterols are formed via the acetate/mevalonate pathway (Lichtenthaler et al. 1997a). Thus, 1-deoxy-D-xylulose and not mevalonate was predominately the precursor of phytol, ß-carotene, and lutein in cell culture of *Catharanthus roseus,* and thus incorporation of deoxyxylulose into sterols (sitosterol) was about 15-fold lower than in into phytol and carotenoids (Arigoni et al. 1997). Also, isoprene emitted by various plant species is formed via the GAP/pyruvate pathway (Lichtenthaler et al. 1997a). It seems that this novel pathway for IPP formation in plastids is a heritage of their prokaryotic, endosymbiotic ancestors (Lichtenthaler et al. 1997a). However, the function and co-operation between the classical and novel pathways is not clear since high activity of HMGR-CoA, enzyme typical for mevalonate pathway, has been localized also in chloroplasts (Kim et al. 1996).

3.3.4 Formation of isoprenoids in tansy

As discussed in chapter 3.2.1, about one hundred volatile compounds have been detected in tansy, many of those have been classified according to their cyclization and functional group (Héthelyi et al. 1991) (Table 7). The possible biosynthetic routes for camphor via bornyl pyrophosphate, bornyl and camphene (Dehal and Croteau 1987; Croteau et al. 1985; Croteau and Felton 1980) and α- and β-thujone via sabinene (Dehal and Croteau 1987; Banthorpe et al. 1978; Banthorpe and Wirz-Justice 1972) have been suggested (Fig. 3).

The existence of the novel IPP biosynthesis in the formation of essential oil of tansy was indicated in an early study which showed that two metabolic pools associated with monoterpene biosynthesis existed and tracers of glucose-[U-^{14}C] were detected equally from two different units of geraniol (Allen et al. 1976). The importance of MVA and IPP in the formation of monoterpenes was demonstrated by incorporating ^{14}C-labelled

substrates into geraniol and nerol, the obligatory precursors for cyclic isothujone (Banthorpe et al. 1978; Banthorpe et al. 1976a). Also, traces of [^{14}C]-labelled α-terpineol and isothujone were detected from carotenoids (Banthorpe et al. 1972).

Table 7. Classification of monoterpenes in tansy according to their cyclization and functional group (Héthelyi et al. 1991)

Cyclization	Classification according to functional groups	
Acyclic	Ketonic constituents:	-
	Alcoholic constituents:	lyratol
		linalool
	Acetatic constituents:	lyratyl acetate
Mono- and bicyclic	Ketonic constituents:	camphor
		carvone
		dihydrocarvone
		piperitone
		thujone
		umbellulone
	Alcoholic constituents:	borneol
		campholenol
		myrtenol
		terpinen-4-ol
	Acetatic constituents:	carveyl acetate
		bornyl acetate
		β-terpinyl acetate
		4-thujen-α-yl-acetate
	Others:	camphene
		1,8-cineole
		pinene
		piperitone
Irregular monoterpenes;	Ketonic constituents:	artemisia ketone
		davanone
	Acetatic constituents:	chrysanthenyl acetate
	Alcoholic constituents:	artemisia alcohol
		chrysanthenol
		yomogi alcohol

In later studies, culture lines of tansy yielded cell-free extracts containing prenyltransferases and an isomerizing system for the conversion of IPP into GPP, neryl pyrophosphate (NPP), and FPP, the activity of which was 3-400 fold higher than activities found from intact plants or up to 90-fold higher than that found from seedlings. Also other enzymes essential for monoterpene synthesis such as kinases, decarboxylases and

isomerases (IPP-DMAPP isomerase) at levels comparable to parental species have been detected (Banthorpe et al. 1986). The activity of prenyl transferase (DMAPP:IPP-dimethylallyl transferase) was observed to vary seasonally (Banthorpe et al 1983) due to the concentration and type of phenols present and the physiological state of the plant. Maximum activity occurred for a short period before the onset of flowering (Banthorpe et al. 1976b).

It was demonstrated that the conversion of geranyl pyrophosphate occurs via an izomerization-cyclization process (Croteau et al. 1985) to (-)-bornyl pyrophosphate and (-)-camphene (Croteau et al. 1990), and that both the isomerization and cyclization take place at the same active site (Croteau et al. 1990). Monoterpenol oxidations may not be catalyzed by group-specific dehydrogenases (Croteau and Felton 1980) but specific dehydrogenases are responsible for example for the biosynthesis of (-)-camphor from borneol and (+)-3-thujone via sabinone in tansy (Dehal and Croteau 1987) (Fig. 3).

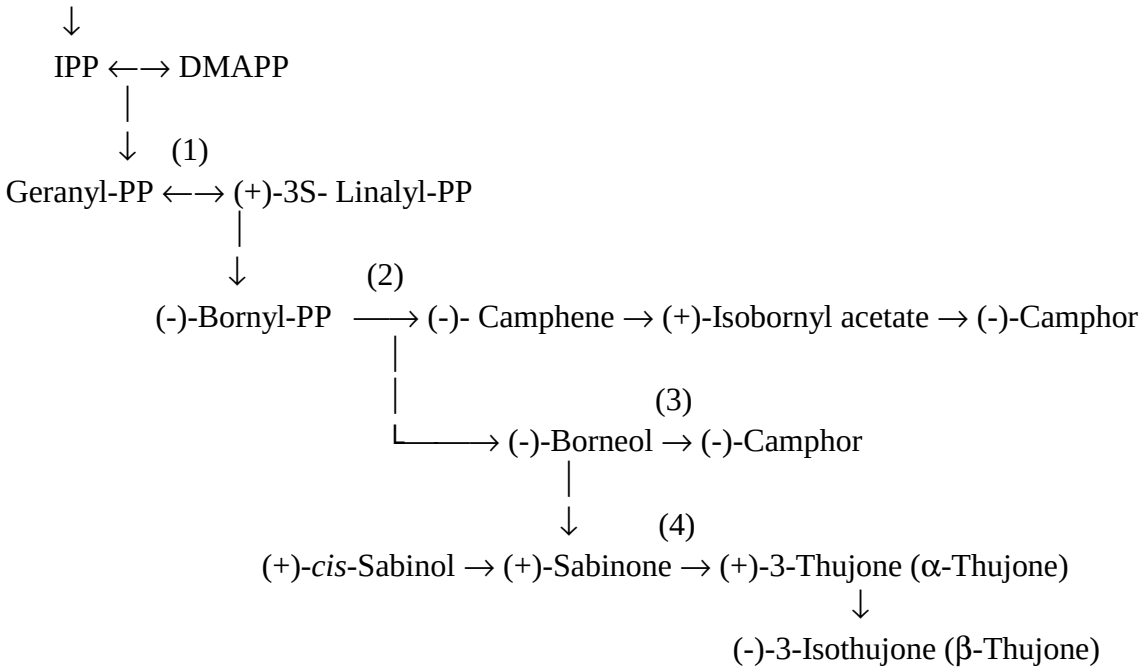

Figure 3. Schematic model for cyclic monoterpene biosynthesis and related enzymes involved in tansy. Isomerization of geranyl-PP to linalyl-PP and following cyclization occur at the same reaction center (Croteau et al. 1990). Enzymes: (1) Isomerization; (2) (Bornyl pyrophosphate cyclase) Monoterpene cyclase; (3) Dehydrogenase; (4) Dehydrogenase. (Modified from Croteau et al. 1990; Dehal and Croteau 1987; Croteau et al. 1985; Croteau and Felton 1980).

3.3.5 Formation of isoprenoids in pyrethrum

The pyrethrins are a mixture of closely related esters derived from chrysanthemum (monocarboxylic) acid, chrysanthemum dicarboxylic acid (pyrethric acid), and from rethrolones (keto-alcohols). (+)-Chrysanthemum acid is the isoprenoid constituent of esters called pyrethrins I (cinerin I, jasmolin I, and pyrethrin I), and similarly (+)-pyrethric

acid is the isoprenoid constituent of pyrethrins II (cinerin II, jasmolin II, and pyrtehrin II) (Godin et al. 1966; Godin et al. 1963; Crowley et al. 1962). The three rethrolones are cinerolone, pyrethrolone (Crowley et al. 1961) and later discovered jasmolone (Crombie 1980).

The biosynthesis of pyrethrins starts from the condensation of two molecules of by an unusual ‘middle-to-tail’ cleavage (Pattenden and Storer 1973), instead of the more normal condensation of one molecule of IPP and one DMAPP by ‘head-to-tail’ cleavage of isoprenoids, into chrysanthemyl alcohol (Godin et al. 1963; Crowley et al. 1962). Oxidation of chrysanthemyl alcohol yields chrysanthemic acid (Crombie 1980) which accumulates also as a glucoside ester (Kueh et al. 1985). The chrysanthemic acid belongs to monoterpenes (Pattenden and Storer 1973) and is structurally analogous to presqualene (Fig. 1), an important precursor in squalene synthesis (Donia et al. 1973; Pattenden and Storer 1973) (Fig. 4).

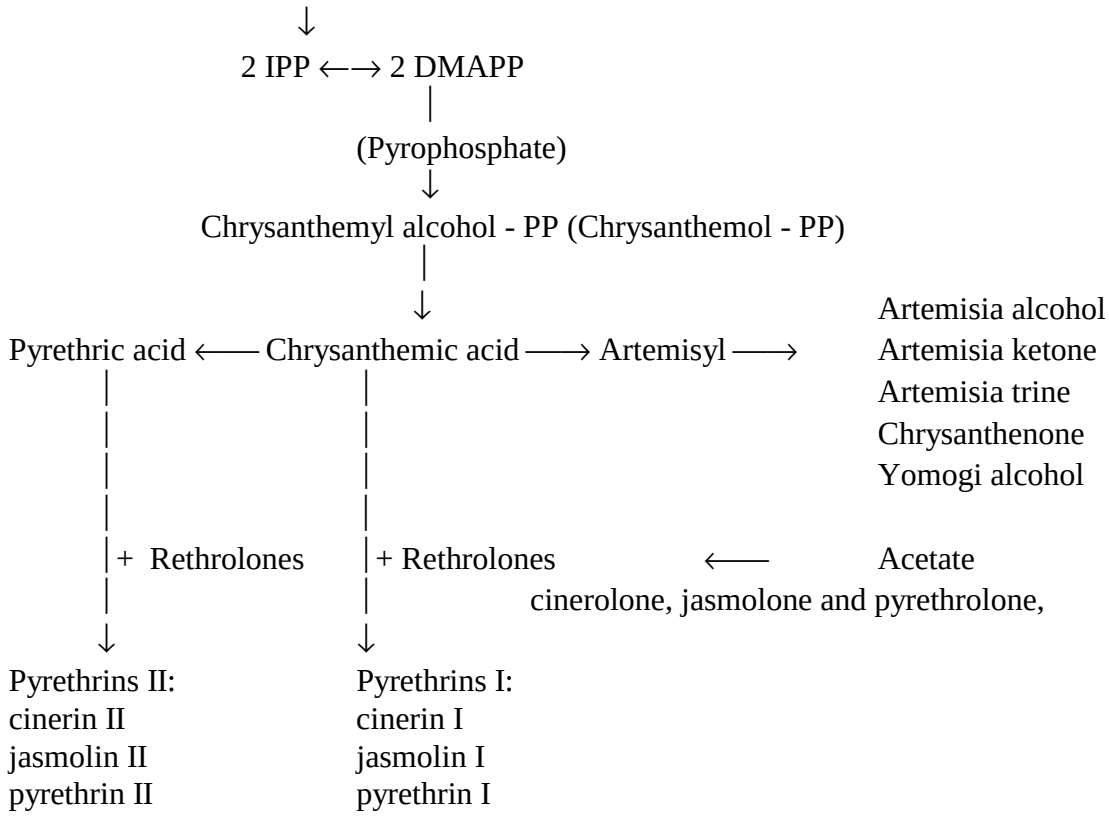

Figure 4. Possible biosynthesis of the pyrethrins in pyrethrum and irregular monoterpenes in tansy (Modified from Zito et al. 1991; Staba and Zito 1985: Greger 1977).

The three esters of pyrethrins I are formed from chrysanthemic acids and the three respective rethrolones, and similarly pyrethrins II are formed from pyrethric acid and the three rethrolones (Kueh et al. 1985; Crombie 1980; Donia et al. 1973; Pattenden and

Storer 1973; Godin et al. 1963; Crowley et al. 1962). The rethrolones (i.e. cinerolone, jasmolone and pyrethrolone) are not formed via mevalonic acid (Crombie 1995; Crowley et al. 1962; Crowley et al. 1961) whereas they are derived most obviously from fatty acid metabolism (Crombie 1995). Thus, the pyrethrins are localized in the achenes of the flower which contain high quantities of linoleic and linolenic acids (Head 1969). Although, the biosynthesis of pyrethrins has been described in detail recently, especially the synthesis of the rethrolones still remains unclear (Crombie 1995). The role of chrysanthemic acid as a precursor for other non-'head-to-tail'-terpenes such as artemisia alcohol, artemisia ketone, artemisia triene, chrysanthenone, yomogi alcohol, lavendulol, and santolina triene, the five first mentioned also detected from tansy (Héthelyi et al. 1994; Collin et al. 1993; Hendriks et al. 1990; Héthelyi et al. 1989; Forsén 1975, Sorsa et al. 1968) have been proposed (Crombie 1995; Boulton et al. 1986; Crombie 1980; Greger 1977; Crombie et al. 1967) (Fig. 4).

Isoprene units have been demonstrated to be important constituents for pyrethrins, because radioactive mevalonic acid incorporated into pyrethrins in 24 hours after feeding isolated ovules with DL-2^{14}C-mevalonic acid (Pattenden and Storer 1973; Crowley et al. 1961; Crowley et al. 1962). The period for which biosynthesis of pyrethrins continued in feeding experiments with [2-^{14}C]-mevalonic acid was dependent on the ripeness and freshness of the ovules (Crowley et al. 1962) which are the major site of pyrethrins synthesized (Crowley et al. 1961). Incorporation of mevalonic acid proceeded into closed ovules as efficiently as into open ones indicating that fertilization did not alter the pyrethrin synthesis (Crowley et al. 1962). Incorporation of [1-^{14}C]-isopentenyl pyrophosphate into chrysanthemyl alcohol, chrysanthemic acid and products from isopentenyl pyrophospate isomerase was demonstrated also in a cell-free homogenate of pyrethrum callus (Zito et al. 1991).

4 *In vitro* establishment of tansy and pyrethrum

4.1 Establishment of tissue culture conditions for recalcitrant species

It has been observed that species belonging, for example, to the family Solaneceae are more adapted to *in vitro* conditions than many other species. Thus, biotechnology as a part of breeding technology was introduced already in the beginning of 1970's to many important species in Solanaceae (Lorenzini 1973; Carlson et al. 1972; Power et al. 1970) whereas the first reports from the most important crops in Asteraceae such as *Chrysanthemum* (Bush and Pueppke 1991; Ledger et al. 1991; von Wordragen et al. 1991; Amagasa and Kameya 1989; Otsuka et al. 1985) and *Helianthus* (Moyne et al. 1989; Schmitz and Schnabl 1989; Moyne et al. 1988; Bohorova et al. 1986; Lenee and Chupeau 1986) date to the end of 1980's. Many of these Asteraceae species are considered as recalcitrant, often due to the lack of knowledge of the species specific conditions needed for successful growth in *in vitro*. Recalcitrance in root or shoot formation or in regeneration may be associated with problems such as endogenous bacterial contamination, vitrification, or tissue browning especially in wild, woody or under-exploited crops (Keskitalo et al. 1998b). As a consequence, cultivation, i.e. growth of species in tissue culture for different biotechnological purposes may only be successful after an *in vitro* 'domestication' period.

4.1.1 Contamination

A critical stage in the introduction of plants to tissue culture is to obtain cultures free of microbial contamination. In spite of the surface sterilization process carried out for explants before culture microbial growth inside the plant cannot be eliminated (Misaghi and Donndelinger 1990; Hennerty et al. 1988). This may be particularly problematic when explants are excised from perennial (Keskitalo et al. 1995; Brunner et al. 1995; Buckley et al. 1995; Savela and Uosukainen 1994) or field grown plants (Peñalver et al. 1994; Savela and Uosukainen 1994; Leben 1972) and transferred to *in vitro* culture.

Contaminants in the xylem vessel (van Doorn et al. 1991) which are protected from surface sterilization (Hallman et al. 1997) are endophytic bacteria detected even in meristem-tip explants (Cassells and Tahmatsidou 1996). Endophytic bacteria have probably evolved a close relationship with their host plant through co-evolutionary processes and may influence plant physiology in ways that have not yet been elucidated (Misaghi and Donndelinger 1990). Inside the plant they have very little microbial competition (Misaghi and Donndelinger 1990) and usually they do not cause visible symptoms to the plant (Hallman et al. 1997; Peñalver et al. 1994). The bacteria may stay latent or symptomless (Peñalver et al. 1994) up to several months after the initiation of culture (Keskitalo et al. 1998b; Reed et al. 1995; Leifert et al. 1992) and may not survive outside the plant tissue (Taber et al. 1991; Trick and Lingens 1985). Endophytic bacteria may even promote beneficial effects for field grown crops (Sturz and Christie 1995), but in stress conditions such as *in vitro* culture, latent endophytic bacteria may become pathogenic and detrimental to the growth and development of the plantlets (Leifert and Waiters 1992; Leifert et al. 1991; Leifert et al. 1989b; Trick and Lingens 1985).

Organic soil amendments have been observed to increase the inoculum for the contamination of plants and thus organic material should be avoided in the preparation of plants for micropropagation (Cassells and Tahmatsidou 1996). Also, explants taken from different locations have been observed to differ in their *in vitro* bacterial contamination (Cassells and Tahmatsidou 1996). It has been demonstrated that many of these non-fastidious bacterial contaminations are environment associated (Cassells and Tahmatsidou 1996), and may be transmitted through water (Seabrook and Farrell 1993; Taber et al. 1991). Bacteria have been detected especially from tissues close to the soil (Fisher et al. 1992; Jacobs et al. 1985). In pyrethrum, more serious bacterial contamination was observed when old flower stems were used as explants for *in vitro* regeneration instead of young flower stems (Roest 1976). Endophytic bacteria have been demonstrated to move within the plant (Chen et al. 1995; Whitesides and Spotts 1991) and tissue contamination may be a consequence of wounding, for example, by infested nematodes (Hallman et al. 1998). Thus, the likelihood of bacterial infection *in vitro* can be reduced by using greenhouse grown starting material.

Most of the plant associated microorganisms detected and identified from *in vitro* cultures have been Gram-negative bacteria (Brunner et al. 1995; Peñalver et al. 1994; Leifert et al. 1989a) some of which may possess resistance to heat (Trick and Lingens 1985). Also filamentous fungi, yeast (Leifert et al. 1994) and Gram-positive bacteria have been identified. The occurrence of the latter increases with time of the *in vitro* culture (Leifert et al. 1989a).

Different antibiotics either alone or in combinations (Keskitalo et al. 1998b; Fellner et al. 1996; Brunner et al. 1995; Reed et al. 1995; Savela and Uosukainen 1994; Kneifel and Leonhardt 1992; Leifert et al. 1992; Leifert et al. 1991) are frequently tested and used for eradication of bacterial contamination. Also constituents in the plant growth media, especially the pH may alter the growth of the microorganism (Leifert et al. 1994; Leifert et al. 1992; Leifert et al. 1989b). Other attempts for elimination of bacterial contamination include stringent surface sterilization methods (Misaghi and Donndelinger 1990), thorough sterilization of laboratory equipment at 140 °C (Trick and Lingens 1985) and prolonged antibiotic treatment and retests during the *in vitro* culture (Reed et al. 1995). Ficoll density gradient centrifugation for protoplasts (Attree and Sheffield 1986) and regeneration from callus (Möllers and Sarkar 1989) have been used to obtain sterile tissues from infected *in vitro* cultures.

The most serious problem for continuos use of antibiotics is the contaminant becoming resistant, although scientific reports of this problem are scare. Bacteria may be associated with plant species (Leifert et al. 1991), which are also sensitive to antibiotics (Keskitalo et al. 1998b; Reed et al. 1995, Savela and Uosulainen 1994; Leifert et al. 1991). Antibiotic at bactericidal concentrations may be toxic to the plant and reduce plant growth (Keskitalo et al. 1998b; Reed et al. 1995; Leifert et al. 1992; Tsang et al. 1989). On the other hand, many antibiotics have enhanced shoot regeneration, shoot development (Yepes and Aldwincle 1994); delayed the loss of regeneration potential (Pius et al. 1993); stimulated callus growth and root formation (Keskitalo and Pehu, unpublished; Keskitalo et al. 1998b; Holford and Newbury 1992); and enhanced plant differentiation from somatic embryos (Eapen and George 1990). Additionally, antibiotics may stimulate enzymes responsible for nitrogen metabolism (Santos and Salema 1989); Hill reaction in chloroplasts, and increase chlorophyll, carotene, and xanthophyll content in leaves (Mukherji and Biswas 1981).

4.1.2 Tissue browning

In vitro tissue browning is a serious problem often associated with woody and perennial species (Choi et al. 1998; Ndoumou et al. 1997; Block and Lankes 1995; El-Hadrami et al. 1993; Bhat and Chandel 1991). Browning is a consequence of many enzymatic reactions where chlorogenic acid (Cheng and Crisosto 1995) or other phenolic compounds are oxidized (Block and Lankes 1995; Dowd and Norton 1995; Bhat and Chandel 1991). The most brown calli showed peroxidase (POD), soluble and bound polyphenol oxidase (PPOS and PPOL), and glutathione reductase (GR) activity. In parallel with the increase of PPOS activity, also superoxide dismutase (SOD) activity has been observed to increase (El-Hadrami et al. 1993). The activity of the latter enzyme was associated with enhanced formation of embryogenic calli (Housti et al. 1992a). The onset of tissue browning was associated with changes in protein pattern, amino acid content, ethylene production and the occurrence of sucrose and accumulation of starch (Linfors et al. 1990).

It is possible, that some external factors trigger stress symptoms such as browning in plant tissue. These factors may be pathogens (Jin et al. 1996) or in some cases even agar (El-Hadrami et al. 1993). High concentration of other medium components such as macrosalts (Choi et al. 1998), auxins (Mohamed and Jayabalan 1996; Baker and Wetzstein 1994), and sucrose (Curtis and Shetty 1996; Rout and Das 1994) have caused browning as well. In

addition, there are some substances including 5,6-Cl_2-IAA (Chen et al. 1992), yeast extracts (Curtis and Shetty 1996), and phloridzin (Block and Lankes 1995) which directly enhance the production of phenolic compounds.

Several factors in the growth and culture conditions of the donor tissues have been tested, that may in later steps of the establishment of tissue cultures cause browning. *In vitro* formation of phenolic acids from explants diminished when donor tissues were preincubated on a medium with (Block and Lankes 1996) or without mineral salts (Parra and Amo-Marco 1996); treated with BA (200 mg l^{-1}) (Rugge et al. 1995); or PVP, ascorbic acid, cysteine, or citric acid (Toth et al. 1994). Accumulation of phenolics is most obviously associated with the developmental stage of the plant and season, which was shown with woody species (Wang et al. 1994). Explants collected from November to February had produced low browning percentages *in vitro*, whereas the browning was at its maximum if explants were collected in April-August (Wang et al. 1994). Differences in browning between varietes and in relation to the size of the explant were also observed (Dalal et al. 1993). The authors concluded that a better way to overcome tissue browning was to optimize growth conditions of the source plants rather than by treating the explants afterwards (Block and Lankes 1996). The most effective antibrowning treatment was growing the source plants in the dark, in the greenhouse, or use of explants from heat-treated or cold-treated source plants (Block and Lankes 1996; Dalal et al. 1993). Simple, but effective inhibition was achieved by sealing the cut ends of explants with paraffin (Bhat and Chandel 1991).

Many medium components have been observed to decrease or eliminate tissue browning such as inclusion of nitrate as a source of nitrogen (Bergmann et al. 1997); increased concentration of phytagel (Li et al. 1997) or using gelrite instead of agar (El-Hadrami et al. 1993). Many authors have also tested phenol traps such as activated charcoal (Prathanturatug et al. 1996); adsorbent resin (Prathanturatug et al. 1996); citric acid (Prathanturatug et al. 1996); cysteine (Pindel and Miczynski 1996); PVP (Prathanturatug et al. 1996; Housti et al. 1992b); and antioxidants such as ascorbic acid (Banerjee et al. 1996; Pindel and Miczynski 1996; Housti et al. 1992b) or glutathione (GSH) (Nomura et al. 1998). In addition, culturing *in vitro* plantlets under low light intensity (Prathanturatug et al. 1996; Phoplonker and Caligari 1993) has given good results.

Tissue browning can also be a serious problem for protoplast cultures. Supplementing the growth medium with antioxidants such as ascorbic acid (Prakash et al. 1997), PVP (Prakash et al. 1997), and charcoal (Reustle and Natter 1994) prevented browning of protoplasts. A similar effect was also achieved by increasing the pH value of the medium (Li et al. 1992). Culturing protoplasts in the dark (Prakash et al. 1997), with mild shaking, in reduced colony density (Hossain et al. 1995), or with addition of fresh medium (Nakano et al. 1995) browning of the cells was prevented.

The initial browning develops later into intense tissue browning and deterioration which is reflected in reduced capacity for protein synthesis and changes in the free amino acid pools and protein patterns (Linfors et al. 1990). Oxidized products, such as quinones, are known to be highly reactive and inhibit enzyme activity leading to the death of the explants (Bhat and Chandel 1991). The toxicity of the oxidized compounds to larvae feeding on a brown callus caused death or reduced growth (Dowd and Norton 1995). As a consequence of browning, tissue senescence (Phoplonker and Caligari 1993), recalcitrance in

embryogenesis and regeneration (Zhong et al. 1995; Rout and Das 1994; Baker and Wetzstein 1993; Housti et al. 1992a; Quinn et al. 1989) have been observed.

4.1.3 Hyperhydricity

Vitrification was first used as a term in conventional plant tissue culture to describe a morphological response of plant tissues to stresses (Franck et al. 1995). The symptoms were water soaked, thick, elongated, wrinkled, curled, brittle and translucent leaves, shoots with shorter internodes (Jones et al. 1993; Debergh et al. 1981), and the low number of shoots/explant (Castro-Concha et al. 1990). However, as a term vitrification is confusing, because in cryobiology vitrification (cryopreservation) describes the process where plant tissue is converted into ice during freezing preservation. Thus, later the same author suggested that the term vitrified should be substituted for hyperhydric (Debergh et al. 1992).

Anatomically hyperhydric leaves have large vacuolated mesophyll cells showing a hypertrophy of the cells (Olmos and Hellin 1998) with large intercellular spaces (Olmos and Hellin 1998; Paques and Boxus 1987) filled with water instead of air (Schloupf et al. 1995). Tissues have less lignin and the vascular system is abnormal (Williams and Raji 1991; Gaspar et al. 1987; Letouzé and Daguin 1987). Leaves have less if any surface wax or the wax has an altered chrystalline structure (Olmos and Hellin 1998), and thin cuticle with a lower level of cutin, pectin and cellulose (Capellades et al. 1990). Several abnormalities have been observed also in hyperhydric cells. The chlorophyll content has been shown to be low (Jones et al. 1993) and to contain abundant plastoglobuli. The guard cells contained high levels of K+ (Olmos and Hellin 1998) and were morphologically abnormal (Olmos and Hellin 1998; Miguens et al. 1993) as well as the stomata them self (Schloupf et al. 1995; Jones et al. 1993; Miguens et al. 1993). Also the photosynthetic rate was lower in hyperhydric cells compared to normal tissue (Jones et al. 1993).

Biochemically the activity of several enzymes is altered in hyperhydric leaves (Gaspar et al. 1987). The activity of superoxide dismutase was high in hyperhydric shoots resulting in hydrogen peroxide (H_20_2) accumulation (Franck et al. 1995). Also, the blockage of the porphyrin pathway (Le Dily et al. 1993) and lack of H_20_2 detoxifying enzymes (catalase, ascorbate peroxidase, and glutathione reductase) (Franck et al. 1995; Le Dily et al. 1993) and a deviation of the nitrogen metabolism induced by NH_3 towards polyamines (Le Dily et al. 1993) caused the formation of toxic oxygen forms (Franck et al. 1995; Le Dily et al. 1993). It is unlikely that nitrate level alone affects hyperhydricity (Brand 1993), although plantlets showed changes in protein synthesis (Gorinova et al. 1993), which correlated both positively and negatively with nitrate content (Brand 1993). It was also observed that habituated (capable to induce callus without exogenous growth substances) and hyperhydric calli resembled each other biochemically (Le Dily et al. 1993).

Development of the morphological symptoms of hyperhydricity depends on many factors. Hyperhydricity has been observed to increase by zeatin (Shibli and Smith 1996), fructose (Druart 1995), thiadiazuron (Sankhla et al.1995), low sucrose concentration (Mondal et al. 1993), BAP (Bouza et al. 1994), and high relative humidity in the culture vessel (Bouza et al. 1992). Visual symptoms occur only for a certain period of time and may vary between species (Turner and Singha 1990). The visual symptoms can be reduced by controlling

various factors. Medium components or growth conditions such as silver nitrate (Madsen et al. 1998), yeast extract (Curtis and Shetty 1996), potato juice (Zhou 1995), increased gelling agent (Curtis and Shetty 1996; Monsalud et al. 1995; Brand 1993; Choudhary et al. 1993; Singha et al. 1990), ABA (Beardmore and Charest 1995; Monsalud et al. 1995), high KNO_3:NH_4Cl_2 ratio (Nagakubo et al. 1993), phloridzin (Jacq et al. 1993), paclobutrazol (Ritchie et a. 1991), increasing gas exchange in the culture vessel (Lakshmanan et al. 1997), and cold (Beardmore and Charest 1995) or reduced temperature (Williams and Raji 1991) treatments and increased Ca concentration (Singha et al. 1990) have decreased the abnormal hyperhydricity of tissues.

4.2 Tissue culture of tansy

Studies on *in vitro* culture of tansy are rather unlimited. Shoot tip culture on Linsmaier-Skoog medium supplemented with auxin and cytokinin was mentioned to be one of the possible propagation methods for tansy (Zámbori-Németh et al. 1987). Different *in vitro* methods such as shoot tip culture; micropropagation; explant-derived callus and suspension cultures; callus derived regeneration; protoplast and protoplast fusion techniques have been developed for tansy (Keskitalo et al. 1999; Keskitalo et al. 1998b; Keskitalo et al. 1995). Pale green or green calli sometimes with red pigmentation have been produced from tansy explants such as leaf, leaf/petiole or leaf tips. Fast growing calli either firm green or friable light green were obtained on medium supplemented with NAA alone or with BAP (Svoboda et al. 1995). Other *in vitro* callus studies of tansy have been established for the production or biochemical investigations of terpenes (Banthorpe and Brown 1990; Banthorpe and Brown 1989; Banthorpe et al. 1986; Banthorpe and Wirz-Justice 1972).

4.3 Tissue culture of pyrethrum

The main objective in pyrethrum breeding has been the increase of pyrethrins content and the improvement of agronomical characters. Conventional breeding methods such as induction of triploids or polyploids (Tuikong 1984); heterosis (Singh and Sharma 1989); hybridization (Singh and Sharma 1989; Parlevliet and Contant 1970; Jones 1968); and clonal selection (Singh and Singh 1996; Singh et al. 1988a; Singh et al. 1987a; Bhat et al. 1985; Parlevliet 1975; Parlevliet and Contant 1970; Parlevliet 1969) have increased the pyrethrins content from less than 1% up to 3% of the DW during the last 30 years (Ian Folder 1996, personal communication). For further improvement of pyrethrum *in vitro* techniques such as axillary shoot cultures, shoot regeneration and callus cultures have been attempted. Many of these experiments have been conducted to study or produce pyretherins in aseptic conditions (Hitmi et al. 1997; Barthomeuf et al. 1996; Dhar and Pal 1993; Nirmala et al. 1992; Rajasekaran et al. 1991; Sarker and Pal 1991; Rajasekaran et al. 1990a; Ravishankar et al. 1989; Kueh et al. 1985; Zieg et al. 1983) although no *in vitro* production method comparable to intact plants has been developed so far.

4.3.1 Micropropagation and shoot tip cultures

Axillary bud development and shoot formation was the highest in a two step culturing program: first shoot tips were cultured on MS medium supplemented with BAP (0.1μM) for 10 days and subsequently transferred to multiplication media supplemented with a higher concentration of BAP (0.9μM) yielding on average 21.5 shoots per explant (Wambugu and Rangan 1981). Pyrethrum shoots were cultured *in vitro* either on liquid or solid media for pyrethrins production (Staba et al. 1984). Rooting was obtained either culturing shoots further on MS medium supplemented with NAA (1.1 μM) (Wambugu and Rangan 1981) or transferring them directly to pots in the greenhouse (Karki and Rajbhandary 1984).

4.3.2 Regeneration from explants

Pyrethrum has been mentioned to be recalcitrant in shoot regeneration (Kueh et al. 1985) especially from leaf explants (Zieg et al. 1983). However, shoot regeneration has been obtained from petiole and leaf explants (Pal and Dhar 1984); leaf derived callus (Paul et al. 1988; Pal and Dhar 1984); young seedlings derived callus (Kueh et al. 1985), and via embryogenesis (Pal 1992). High regeneration potential has been observed to be associated with high pyrethrins content (Paul et al. 1988; Zieg et al. 1983). Regeneration has been initiated from leaf-derived callus on medium supplemented with BAP (Dhar and Pal 1993; Paul et al. 1988; Zieg et al. 1983); BAP + NAA, BAP + GA (Paul et al. 1988), kinetin + IAA, or BAP + IAA (Kueh et al. 1985). Shoots regenerated from leaf explants were cytologically homogeneous and identical in terms of their ploidy level compared to the donor plants. However, plants regenerated from petiole tissue varied in chromosome number suggesting that petiole explants could be exploited to raise new cytotypes of pyrethrum plants (Pal and Dhar 1984).

Regeneration of adventious roots from peduncle explants of pyrethrum has been studied in detail (Roest 1976). To obtain the highest number of roots the growth conditions should be as follows: peduncles excised at flowering stage, cut in 1.5 cm long pieces and wounded over the entire length of the explants, placed horizontally with the wounded side on medium supplemented with Knop medium of half strength, sucrose (2%), and IBA. The concentration of IBA for the initiation of roots in the first week should be 0.01 mg l^{-1} g after which explants should be transferred to auxin free medium. The highest number of roots have been obtained culturing the explants in the dark first two weeks at 13°C and subsequently cultured at 20°C (Roest 1976). Roots have been initiated also from explants when culturing on media supplemented with 2,4-D (Dhar and Pal 1993); 2,4-D and BAP (Zieg et al. 1983); or IAA (Kueh et al. 1985) and placed in the dark at 25 °C (Kueh et al. 1985).

4.3.3 Establishment of callus cultures

Calli have been initiated from leaf (Barthomeuf et al. 1996; Rajasekaran et al. 1996; Nirmala et al. 1992; Rajasekaran et al. 1991; Sarker and Pal 1991; Rajasekaran et al. 1990a; Rajasekaran et al. 1990b; Ravishankar et al. 1989; Paul et al. 1988; Kueh et al. 1985; Pal and Dhar 1984; Zieg et al. 1983), petiole (Sarker and Pal 1991; Kueh et al.

1985; Pal and Dhar 1984), disc florets (Barthomeuf et al. 1996; Zieg et al. 1983), flower buds (Barthomeuf et al. 1996); flower stems (Barthomeuf et al. 1996; Zieg et al. 1983), achene wall, or receptaculum (Zieg et al. 1983) explants of pyrethrum on MS media containing 2,4-D and BAP (Dhar and Pal 1993; Sarker and Pal 1991: Zieg et al. 1983); 2,4-D and kinetin (Rajasekaran et al. 1996; Rajasekaran et al. 1991; Rajasekaran et al. 1990a; Ravishankar et al. 1989; Kueh 1985) or ANA (ß-naphtoxy acetic acid) and BAP (Barthomeuf et al. 1996). About 14- and 11-fold increase in callus growth has been observed following an increase in the concentration of thiamine (to 0.4 mg l^{-1}) (Kueh et al. 1985) or supplement of CNP nutrients at a concentration of 2:1:2 (Rajasekaran et al. 1990a), respectively. Also, callus growth was more consistent if explants were cultured on half-strength, revised, tobacco MS medium (Zieg et al. 1983). In contrast, callus growth of pyrethrum was reduced about 40% if the concentration of formaldehyde (an antimicrobial agent) was higher than 0.025% (Nirmala et al. 1992). The chromosome number of calli grown less than 5 passages was consistently diploid (2n=18) whereas polyploid cells appeared in long term cultures (Pal and Dhar 1984). Embryogenic calli derived from leaf explants was only induced in sectors of the calli where diploid (2n=18) chromosome number predominated. The number of chromosomes varied from 12-72 in the non-embryogenic sectors (Pal 1992). No correlation between pyrethrins yield and the growth rate of the callus was observed (Sarker and Pal 1991).

4.3.4 Commercial interest for pyrethrins production *in vitro*

In 1984 a patent for the enzymatic synthesis of pyrethrins was granted to an American company, McLaughlin Gormley King, in Minnesota. The patent was mentioned to describe a process for the production of radioactively labeled pyrethrins, although the composition of the cell-free homogenate regarding enzymes and cofactors has not been defined in the patent (Jovetiç 1994). A U.S-based plant biotechnology company, AgriDyne Technologies Inc., Utah is studying pyrethrins production by genetically engineered micro-organisms. As reviewed by previous authors (Jovetiç and de Gooijer 1995) this company has cloned a gene coding for the chrysanthemyl diphosphate synthase and is testing its activity and level of production in microorganisms. In 1992 AgriDyne Technologies in Salt Lake City (Utah, USA) announced to invest US$ 3 million in the next 3 years to develop a genetically engineered pyrethrin product (Shand 1992). It was speculated that if the company will succeed to develop a microorganism producing pyrethrins, it could be devastating especially to many of the small farmers in South-America, India, and Africa who still produce major part of the US$ 100-400 million annual trade of natural pyrethrins (Shand 1992; Sihanya 1992). However, many consider that microorganism-based pyrethrins are unlikely to reach the market in the near future (Sihanya 1992).

4.4 Tissue culture of other species in *Tanacetum* and *Chrysanthemum* genera

There are only a few other species in the genus *Tanacetum* which have been introduced to *in vitro* culture. Recently, different *in vitro* techniques such as callus (Brown et al. 1996; Banthorpe and Brown 1989), suspension (Brown et al. 1996; Banthorpe and Brown 1990), shoot regeneration (Brown et al. 1996), axenic shoot production (Stojakowska and Kisiel 1997; Brown et al. 1996), and *Agrobacterium rhizogenes* transformation (Kisiel and Stojakowska 1997; Stojakowska and Kisiel 1997) have been developed for anti-migraine

compound (parthenolide) accumulating feverfew (*Tanacetum parthenium*). Regeneration method from achenes and petals of pyrethrins producing *Tanacetum coccineum* (*Chrysanthemum coccineum*) have also been developed (Fujii and Shimixu 1990).

Tissue culture methods have been applied to several species of the genus *Chrysanthemum*, which consists of many commercially important cut flowers. These include: *Chrysanthemum coronarium* (Lee et al. 1997), *Chrysanthemum x hortorum* (Sauvadet et al. 1990), *Chrysanthemum indicum* (Kim and Kim 1998; Ledger et al. 1991; Okamura et al. 1984), *Chrysanthemum morifolium* (syn. *Dendranthema grandiflora*) (Khehra et al. 1995; Aribaud et al. 1995; Aribaud 1994; Urban et al. 1994; Lowe et al. 1993; Malaure et al. 1991; Bhattacharya et al. 1990; Kaul et al. 1990; Lu et al. 1990; Amagasa and Kameya 1989; Otsuka et al. 1985), and *Dendranthema zawadskii x D. grandiflora* (Lindsay and Ledger 1993).

5 Protoplast technique as a tool for metabolic engineering of the isoprenoid pathway

5.1 Protoplast technique in *Tanacetum* and *Chrysanthemum* genera

Plant protoplasts are cells from which the cell wall has been removed enzymatically, but they retain all the intracellular organelles including the nucleus. The protoplast is capable of expressing totipotency through cell division and regeneration into a plant. Regeneration from protoplasts is possible for a large number of species, many of important agronomic value (Roest and Gilisson 1993; Roest and Gilisson 1989), which makes protoplast cultures into an interesting alternative for genetic engineering and plant improvement. Single protoplasts have been used as material inducing somaclonal variation following mass regeneration of plants. In addition, protoplast culture is a valuable tool for a variety of studies, including uptake of exogenously supplied materials, such as viruses (Matsunaga et al. 1992), and macromolecules like DNA and gene (Kârle et al. 1993; Kirches et al. 1991). Also physiological (Okamura et al. 1984) and metabolic investigations (Szakiel and Janiszowska 1992; Bagni and Pistocchi 1990), ultrastructural studies, and the isolation of subcellular components, including nuclei, chromosomes, and vacuoles can be studied using protoplasts.

Protoplast isolation is theoretically a simple process, which may, however, include numerous problematic steps particularly when applying the technique to a new plant species. For example, in the family Asteraceae only a few genera have been successfully regenerated into plants (Table 8). Previous protoplast culture experiments carried out on pyrethrum or related species in *Chrysanthemum* have ceased at the callus stage (Amagasa and Kameya 1989; Malaure et al. 1989; Okamura et al. 1984; Schum and Preil 1981), with the exception of *Dendranthema zawadskii* x *D. grandiflora* (syn *Chrysanthemum*) (Lindsay and Ledger 1993), *C. x hortorum* (Sauvadet et al. 1990), and *C. morifolium* (Otsuka et al. 1985) (Table 8).

Table 8. Application of protoplast technique in species belonging to the family Asteraceae

Species	Source	Type of regeneration	Reference
Artemisia sphaerocephala	hypocotyls	callus	Xu and Jia 1996
Artemisia vulgaris	mesophyll		Okamura et al. 1984
Brachycome iberidifolia	leaves	shoots	Malaure et al. 1990
Callistephus chinensis	mesophyll	shoots	Pillai et al. 1990
Centaurea cyanus	mesophyll	shoots	Pillai et al. 1990
Chrysanthemum x hortorum	mesophyll	shoot	Sauvadet et al. 1990
Chrysanthemum indicum	mesophyll		Okamura et al. 1984
Chrysanthemum morifolium	mesophyll	callus/ shoots	Amagasa and Kameya 1989/ Otsuka et al. 1985
Cichorium intybus x endiva L.	mesophyll	shoots	Sidikou-Seyni et al. 1992
Cichorium intybus	mesophyll	normal and albino shoots	Varotto et al. 1997; Vermeulen et al. 1993
Cyanara scolymus L.	suspension	callus	Ordas et al. 1991
Dendranthema (syn *Chrysanthemum*) *zawadskii*	mesophyll		Okamura et al. 1984
Dendranthema zawadskii x D. grandiflora	leaves	shoot	Lindsay and Ledger 1993
Felicia bergeriana	leaves	shoots	Malaure et al. 1990
Helianthus annuus	cotyledon hypocotyl callus	shoots, callus formation from callus-derived protoplasts	Santos and Caldeira 1998; Laparra et al. 1997; Wingender et al. 1996; Petitprez et al. 1995; Trabace et al. 1995; Krasnyanski and Menczel 1993; Burrus et al. 1991
Helianthus debilis	hypocotyl	callus	Chanabe et al. 1991; Bohorova et al. 1990
Helianthus divaricatus	leaves		Bohorova et al. 1990
Helianthus gigantheus	mesophyll	shoots	Krasnyanski et al. 1992
Helianthus maximiliani	mesophyll	shoots	Polgár and Krasnyanski 1992; Bohorova et al. 1990
Helianthus mollis	leaves	colonies	Bohorova et al. 1990
Helianthus nuttalli	leaves	colonies	Bohorova et al. 1990
Helianthus petiolaris	hypocotyl	shoots	Chanabe et al. 1991
Helianthus praecox	roots	callus	Bohorova et al. 1990; Bohorova et al. 1986
Helianthus rigidus	hypocotyl	callus	Bohorova et al. 1986; Chanabe et al. 1991
Helianthus scaberimus	cotyledons	callus	Bohorova et al. 1986
Lactuca perennis	leaves	shoots	Webb et al. 1994
Lactuca saligna	leaves	shoots	Brown et al. 1987
Pelargonium x domesticum	callus	shoots	Dunbar and Stephens 1991
Petasites japonicus	mesophyll	shoots	Yabe et al. 1986
Rudbeckia hirta	leaves, cotyledons	shoots	Al-Atabee and Power 1987
Senecio x hybridus	mesophyll	shoots	Pillai et al. 1990
Senecio vulgaris	mesophyll	shoots	Binding and Nehls 1980
Tanacetum cinerariifolium	leaves	callus	Malaure et al. 1989
Tanacetum vulgare	mesophyll	callus, root and abnormal shoot formation	Keskitalo et al. 1995

One of the most important prerequisites for successful isolation of protoplasts is that the plant tissue used is suitable donor material (Lindsay and Ledger 1993). Bacterial contamination, tissue browning, and hyperhydricity, as discussed in chapter 4, are some of the harmful factors making candidate donor tissues unsuitable for protoplast isolation and culture. The aim is to isolate protoplasts from fast dividing cells, appreciating that the conditions facilitating cell division may be species specific (Geng-Guang 1996; Lindsay and Ledger 1993).

Protoplast isolation from species in *Tanacetum* and *Chrysanthemum* have been carried out from different donor tissues including callus (Keskitalo and Pehu, unpublished), mesophyll cells of leaves (Keskitalo et al. 1995; Lindsay and Ledger 1993; Sauvadet et al. 1990; Amagasa and Kameya 1989; Malaure et al. 1989; Otsuka et al. 1985; Okamura et al. 1984), *de novo* regenerated shoots (Lindsay and Ledger 1993), and leaves of greenhouse grown plants (Schum and Preil 1981). Usually the viability of protoplasts has been higher if donor plants have been cultured *in vitro* instead of *in vivo* in the greenhouse (Okamura et al. 1984).

In *Dendranthema* (syn *Chrysanthemum)*, the viability of isolated protoplasts maintained high still two days after isolation if the protoplats were isolated from shoots able to *de novo* regeneration (85%) rather than from *in vitro* grown shoot tips (45%) (Lindsay and Ledger 1993). Protoplasts derived from *de novo* regenerated shoots contained less chlorophyll and developed dense cytoplasm within two days (Lindsay and Ledger 1993).

In addition to the source tissue, the protoplast isolation and culture process itself influences cell division and regeneration. Commonly, the tissue for protoplast isolation is first sliced into small strips and incubated for several hours in relatively high concentration of cellulase, hemicellulase, and/or pectinase for the digestion of the plant cell wall (Lindsay and Ledger 1993; Amagasa and Kameya 1989; Malaure et al. 1989; Otsuka et al. 1985; Schum and Preil 1981). Besides the enzymes, an important factor facilitating healthy protoplast isolation is the osmolarity of the isolation and culture medium (Malaure et. al. 1989). Osmolarity, as most of the details in protoplast technology is in most cases empirically tested. During protoplast isolation metabolizing (sucrose) and/or non-metabolizing sugars (mannitol, sorbitol) (Sauvadet et al. 1990; Amagasa and Kameya 1989; Malaure et al. 1989; Otsuka et al. 1985; Okamura et al. 1984; Schum and Preil 1981) are used as osmotica. After the incubation period, viable protoplasts are separated from debris of cell wall material, dead cells, and various metabolites by several washes and tested for the viability of protoplasts.

Protoplast plating density is another critical factor. The plating density of 5×10^4 ml^{-1} was observed to be optimum for *C. x hortorum* (Sauvadet et al. 1990) but it varies between species (Keskitalo et al. 1995). Several recipes for media composition for protoplast culture of *Chrysanthemum* species including the concentration of sugars, macro- and micronutrients, vitamins, hormones, and gelling agents have been reported (Lindsay and Ledger 1993; Sauvadet et al. 1990; Malaure et al. 1989; Okamura et al. 1984). In many cases the most effective procedures have been empirically optimized. Particularly, NH_4 as a source of nitrogen has been shown to inhibit cell division in *Chrysanthemum* (Sauvadet et al. 1990; Amagasa and Kameya 1989; Otsuka et al. 1985; Okamura et al. 1984), while it was stimulated by $Ca(NO_3)_2 \cdot 4H_20$ (Amagasa and Kameya 1989). It was observed that the division frequency of protoplasts of *Chrysanthemum indicum* and *Chrysanthemum zawadskii* was the highest (20%) when protoplasts were cultured without ammonium ions (Okamura et al. 1984). Also high auxin concentration in the initiation medium facilitated rapid cell division (Keskitalo et al. 1995; Lindsay and Ledger 1993; Sauvadet et al. 1990; Otsuka et al. 1985). For *Helianthus annuus* medium solidified with agarose (Wingender et al. 1996; Krasnyanski and Menczel 1993; Fischer et al. 1992; Fischer and Hahne 1992; Krasnyanski et al. 1992) and supplemented with high cytokinin/auxin ratio (Wingender et al. 1996) was essential for embryogenesis and morphogenesis, respectively. During the culture, the concentration and composition of media components are gradually changed to meet the requirements of the cells which start to develop the cell wall around the

protoplasts few days after isolation. Similarly, as protoplast isolation, also the media and environmental requirements during the subsequent protoplast culture may depend on the species (Amagasa and Kameya 1989; Okamura et al. 1984) or even on the genotype (Sauvadet et al. 1990; Malaure et al. 1989; Otsuka et al. 1985).

Protoplast-derived microcolonies are microscopically visible in a few weeks and visually around one month after protoplast isolation. However, quite a wide variation occurs in the developmental speed between species, because for pyrethrum it took one month to reach 8-cell stage (Malaure et al. 1989) whereas at the same time *Chrysanthemum x hortorum* had already developed into microcalli (Sauvadet et al. 1990). In *Drendanthema* the initial division frequency was about eight-fold higher when protoplasts were isolated from *de novo* regenerated shoots instead of *in vitro* grown shoots which were not selected for their regeneration capacity (Lindsay and Ledger 1993). Protoplast-derived callus has been obtained only from a few species in *Chrysanthemum* (Amagasa and Kameya 1989; Malaure et al. 1989; Okamura et al. 1984) whereas *Dendranthema* was successfully regenerated into shoots (Lindsay and Ledger 1993) (Table 8).

Different factors affecting division and regeneration ability of protoplasts isolated from other species in the Asteraceae family have been studied. Protoplasts from dark grown seedlings of *Helianthus annuus* started to divide earlier and with higher frequency compared to seedlings grown in light (Geng-Guang 1996). *Lactuca sativa* protoplasts underwent progressive senescence and lost their viability when incubated in mannitol (Oh et al. 1994). Hypocotyl of sunflower has been one of the most suitable sources for protoplasts isolation. Thus, hypocotyl protoplasts divided symmetrically to form loosely associated microcolonies when cultured in liquid medium, whereas embedded in agarose they divided asymmetrically and gave rise to embryo-like structures (Caumont et al. 1997; Petitprez et al. 1995; Krasnyanski et al. 1992; Fischer et al. 1992). Protoplasts derived from cortical and medullar cells of sunflower hypocotyls had the highest division potential compared to cells from epidermis (Petitprez et al. 1995). This may have caused the formation of protoplasts into two types of tissues. Regeneration was observed from tissues accumulating starch (Laparra et al. 1997). Callus derived from sunflower mesophyll protoplasts differed in its embryogenic ability. Callus having little embryogenicity consisted of small groups of meristematic and vacuolated cells, characterized by incomplete division. In contrast, callus having higher embryogenicity lacked incomplete cell partitioning and showed formation of organized regions such as tracheidals (von Keller et al. 1994). Also, the DNA content of the protoplasts was found to reflect that of the donor cells. Most of the protoplasts from the cortical or medullar parenchyma could be considered as 2C, while protoplasts from the epidermal cells had a lower DNA level (Petitprez et al. 1995).

5.2 Protoplast fusion in Asteraceae

A further application of protoplast technology is protoplast fusion which allows combination of desirable plant traits from two species to one plant which would not be possible by sexual crossing (Krasnyanski and Menczel 1995; Maisonneuve et al. 1995; Chupeau et al. 1994; Rambaud and Vasseur 1994; Matsumoto 1991; Al-Atabee et al. 1990). Only a few fusions have been reported from Asteraceae (Table 9). However, in spite of the difficulties existing in this technology, even protoplasts of barley and carrot

have been fused and regenerated into somatic hybrids (Kisaka et al. 1997). Protoplast fusion is also an alternative method for the production of polyploids in some species (Rambaud et al. 1992).

Table 9. Application of protoplast fusion in species belonging to the Asteraceae family

Species	Source	Type of regeneration	References
Cichorium intybus x *C. intybus*	leaves	tetraploid plants	Rambaud et al. 1992
Cichorium intybus x Helianthus annuus	mesophyll hypocotyl	male sterile plants	Rambaud and Vasseur 1994
Helianthus annuus x *H. annuus*	mesophyll, hypocotyl	sterile plants	Trabace et al. 1996; von Keller et al. 1995; Barth et al. 1993; Biedinger and Schnabl 1991
Helianthus annuus x *H. giganteus*	hypocotyl, mesophyll	fertile hybrids plants	Krasnyanski and Menczel 1995
Lactuca sativa x *L. virosa*	leaves	sterile hybrids plants	Matsumoto 1991
Lactuca sativa x L. tatarica	leaflets	climatic sensitive hybrids	Chupeau et al. 1994; Maisonneuve et al. 1995
Lactuca sativa x L. perennis	leaflets	hybrid plants	Maisonneuve et al. 1995
Rudbeckia hirta x *R. laciniata*	leaves, callus	somatic hybrid plants	Al-Atabee et al. 1990
Senecio fuchsii x *S. jacobaea*		somatic hybrids and cybrids plants	Wang and Binding 1993
Tanacetum vulgare x T. cinerariifolium	mesophyll	callus	Keskitalo et al. 1999

In some species fusion can occur spontaneously when protoplasts are brought in close contact (Barth et al. 1993), but in most cases specialized methods must be used. Before fusion, protoplasts from one species can be treated with iodoacetic acid to render them unable to divide (Krasnyanski and Menczel 1995; Matsumoto 1991) which can be used as a selective marker. Polyethylene glycol (PEG) has been used as a fusogen in chemical fusions in Asteraceae (Trabace et al. 1996; Krasnyanski and Menczel 1995; Chupeau et al. 1994; Rambaud and Vasseur 1994; Rambaud et al. 1992). Cells have been fused also using high pH, and high calsium concentration in conjunction with PEG treatment. Firstly, the isolated and washed protoplasts of the two donors are mixed in equal volume and agglutinated followed by membrane fusion by the fusion agent, PEG. It was observed that protoplasts from *Helianthus* were more sensitive to PEG than those from other species. The problem was overcome by reducing the PEG concentration to ¾ of the initial concentration (Krasnyanski and Menczel 1995). The mechanism of PEG is not precisely known, but it most probably decreases the hydrophobic stabilization of the lipid bilayers due to immobilization of water molecules in the vicinity of the cell membrane. After the

membrane fusion follows a withdrawal of the fusogen with a mixture of buffer and protoplast culture medium by which the membranes of the protoplasts are restabilized. In many cases the plating efficiency after PEG treatment has varied from 0% to 40% of initially plated protoplasts in spite of the different optimization procedures (Chupeau 1994) indicating protoplast sensitivity to various still unknown factors.

Only a few examples of electrically fused protoplasts in the Asteracea family have been reported which have been carried out within the genera *Helianthus* (von Keller et al. 1997; Barth et al. 1993) and *Lactuca* (Maisonneuve et al. 1995). In electrofusion, an equally mixed suspension of protoplasts of both parental species is placed in a fusion chamber between two metal electrodes. Before fusion the protoplasts are brought to a close membrane contact by dielectrophoresis by an alternating current (AC). After this the membranes become differentially charged and as a consequence, protoplasts tend to line up as pearl chains. Membrane breakdown and fusion are achieved subsequently by applying at least one, but sometimes two, direct current pulses (DC). After the membrane breakdown an immediate cessation for 0.5-5 min of cytoplasmic streaming was observed (von Keller et al. 1995). The creation of viable fusion products requires not only membrane fusion but also complete reorganization of the cytoplasmic material including the dislocation of the nuclei (von Keller et al. 1995). Electrical treatment of fusion cells may even stimulate cell wall formation and cell division compared to not electrically treated protoplasts (von Keller et al. 1997; Phansiri et al. 1994). In contrast, damages such as degradation of membrane lipids due to electrofusion may cause increased ethane production and decreased division rate of cells (Biedinger and Schnabl 1991).

5.3 Characterization of fusion products

Both chemical and electrofusion are mass fusions, because they may consist of fusions between two or more protoplasts from either of the parental tissues (Matsumoto 1991). If the fusion involves two nuclei, it is called a heterocaryon (Rambaud and Vasseur 1994), in contrast to a hybrid with combined nuclei (von Keller et al. 1995). In some cases asymmetric hybrids are formed where chromosome elimination has followed the fusion process, and thus the nucleus in the hybrid cell is not composed equally from both of the parents (Wang and Binding 1993; Matsumoto 1991). In contrast to asymmetric hybrids, symmetric hybrids contain the complete chromosome complements from both of the donor species (Krasnyanski and Menczel 1995; Matsumoto 1991; Al-Atabee et al. 1990).

One of the most interesting advantages of protoplast fusion over sexual hybridization is the possibility to combine cytoplasmic genomes such as mitochondria and chloroplasts in the somatic hybrid (Trabace et al. 1996; Rambaud and Vasseur 1994; Wang and Binding 1993). In many cases the cytoplasmic pattern of somatic hybrid is not equally combined from the parental tissues (Rambaud and Vasseur 1994; Wang and Binding 1993; Al-Atabe et al. 1990). Whether the nuclear or cytoplasmic genome is derived from different parents, the fusion product is called a cybrid (Wang and Binding 1993). Production of cybrids is an important result of protoplasts fusion technique, because many important characters such as cytoplasmic male sterility (Cardi and Earle 1997; Rambaud et al. 1997; Rambaud and Vasseur 1994), herbicide (Thomzik and Hain 1988) and antibiotic resistance (Menczel et al. 1981) are coded for by cytoplasmic genes, and cannot be transferred to a hybrid by the conventional sexual crossing method.

Selection and analyzes for the reinforcement of the hybridity are important steps in the protoplast fusion technique. Selection of plant heterocaryons emitting dual green and red fluorescence by flow cytometry is a convenient method (Hammatt et al. 1990), although requires necessary equipment. To use an antibiotic resistant parent reduces the possibility of non-hybrid cells to grow in the selection medium already at an early stage (Chupeau et al. 1994). Characterization of nuclear hybridity at callus stage (Keskitalo et al. 1999; Trabace et al. 1996; Barth et al. 1993) reinforces the hybrid status before possible chromosome elimination occurs occasionally during the progeny of the plant (Rambaud and Vasseur 1994). Whether the hybrids are selected early or not, regenerated plants (Krasnyanski and Menczel 1995; Maisonneuve et al. 1995; Chupeau et al. 1994; Rambaud and Vasseur 1994) are frequently tested for hybridity. To evaluate fusion products in the Asteraceae family morphological characters such as hairiness (Matsumoto 1991), pigmentation (Wang and Binding 1993; Al-Atabee et al. 1990), flower color (Chupeau et al. 1994), pollen viability (Al-atabee et al. 1990), the regeneration type (Al-Atabee et al. 1990), regeneration ability (Matsumoto 1991), vigor (Matsumoto 1991) and the ability of embryogenic development contributed by either of the parent species (Krasnyanski and Menczel 1995) have been employed.

Hybridity can also be studied at the nucleic acid level. The analyses of nuclear DNA content allows for the quantification of the genome, but does not give information of the origin of the DNA (Keskitalo et al. 1999). Other DNA techniques such as RAPDs (Keskitalo et al. 1999; Trabace et al. 1996; Maisonneuve et al. 1995; Chupeau et al. 1994) and RFLP analyses (Wang and Binding 1993), Southern hybrization using species specific probes (Rambaud et al. 1997), glucosephosphate isomerase (Barth et al. 1993) and chloroplast DNA (coDNA) restriction digests (Al-Atabee et al. 1990) yield more information on the origin of the DNA. Hybridity of fusion products among *Helianthus* (Krasnyanski and Menczel 1995; Trabace et al. 1995), *Rudbeckia* (Al-Atabee et al. 1990), *Lactuca* (Matsumoto 1991), and *Senecio* (Wang and Binding 1993) was confirmed by chromosome counting and by the analysis of esterase isozymes. In most of the experiments reported from the Asteraceae family, the problem of sterility in the hybrid is frequently stated in line with the fusion between *Helianthus annuus* and *Helianthus giganteus* (Krasnyanski and Menczel 1995) and *Cichorium x Helianthus* (Rambaud and Vasseur 1994); and among the genera *Lactuca* (Maisonneuve et al. 1995; Chupeau et al. 1994; Matsumoto 1991), and *Helianthus* (Trabace et al. 1996). Chloroplast counts of stomatal guard cells of the intraspecific fusion products of *Cichorium intybus* revealed two types of plants. The first type contained less than 18-20 chloroplasts being most probably a diploid, whereas the other type contained more than 23-24 chloroplasts and was presumed to be tetraploid. In culture the tetraploid *Cichorium* plantlets had bigger roots and larger dark green leaves than the diploids (Rambaud et al. 1992).

5.4 Protoplast fusion as a tool for metabolic engineering

While secondary metabolites can be used as a test method for somatic hybridization it can also be the goal of an entire fusion program. Protoplast fusion has been classically used for crop improvement in traits such as pest and disease resistance (Krasnyanski et al. 1998; Rokka 1998; Matsumoto 1991), cold tolerance (Sigareva and Earle 1997) and transfer of CMS (Cardi and Earle 1997; Rambaud et al. 1997). However, somatic hybridization can also be a novel approach in metabolic engineering as has recently been demonstrated in interspecific hybrids of *Solanum* producing novel alkaloids (Laurila et al. 1998). The possibilities of modifying isoprenoid metabolism is not only for increasing the production of commercially interesting products, but also in connection with the production of new compounds, resistance of plants against pests, the quality of food and ornamental value (Verpoorte et al. 1996). In spite of the molecular and biochemical knowledge of pathways leading to secondary metabolites such as isoprenoids, there is still great uncertainty on the regulation of the pathway. On the other hand, to alter biochemical pathways for example by gene tranformation we should be able to manipulate the function of many enzymes involved in the production of a particular metabolite as well as increase the flux from primary metabolism (Blakeley and Dennis 1993). On the other hand, besides the obvious importance of HMGR-CoA for isoprenoid pathway, there is still little knowledge of the possible other rate limiting enzymes, for example in pyrethrins production.

5.5 Possible routes for manipulating the isoprenoid pathway through protoplast fusion

5.5.1 Somaclonal variation

In addition to the process of nuclear and cytoplasmic hybridization in fusion, also other DNA rearrangements can occur resulting in somaclonal variation (Chaput et al. 1996). The effect of somaclonal variation in terpene composition has been demonstrated with a few species. Protoplast derived *Mentha x piperita* shoots yielded less menthol and menthone but more carvone compared to the control plants (Chaput et al. 1996). Several *in vitro* derived somaclones of Japanese mint (*Mentha arvensis* L. var *piperascens* Holmes) had higher menthol content and improved agronomic or essential oil characters compared to *in vivo* grown plants (Kukreja et al. 1992; Kukreja et al. 1991). Plants with improved oil quality, possessing higher levels of desirable constituents such as citronellal, citronellol, geraniol and geranyl acetate were observed also in an aromatic grass, *Cymbopogon winterianus* Jowitt as a consequence of somaclonal variation (Mathur et al. 1988).

5.5.2 Polyploidization

Protoplast fusion is an alternative technique for chromosome doubling for some species (Rambaud et al. 1992). Often the polyploid plants are bigger due to increased complement of the chromosomes (Gao et al. 1996; Rambaud et al. 1992; Lavania 1988), the phenomenon which may enhance the accumulation of isoprenoids. This was the case when artificial autotetraploids were produced by colchicine treatment in the essential oil bearing vetiver grass (*Vetiveria zizanioides* L. Nash). Tetraploids were vigorous with thicker and longer roots, and the percent of essential oil improved from 0.98% to 1.4% in freshly

harvested roots of tetraploid plants compared to the control (Lavania 1988). Similarly, also roots of a diploid medicinal plant *Salvia miltiorrhiza* contained higher amounts of tanshinones as a result of colchicine treatment due to the autotetraploid chromosome complement (Gao et al. 1996).

5.5.3 Cellular compartmentalization of isoprenoid pathway

In addition to the nucleus, the fusion partner origin of the cellular compartments, most importantly the mitochondria and chloroplasts can vary in the hybrid cell as a result of somatic hybridization (Rambaud et al. 1997). This possibility is particularly important when cytoplasmically controlled characters such as CMS (Cardi and Earle 1997; Rambaud et al. 1997) and chemical resistance (Thomzik and Hain 1988; Menczel et al. 1981) are the traits of interest. It has been shown that at least part of the mevalonic acid pathway (Zhu et al. 1997; McCaskill and Croteau 1995) and even the entire GAP/pyruvate pathway are functional in the plastids (Bouvier et al. 1998; Duvold et al. 1997; Lichtenthaler et al. 1997a;. Lichtenthaler et al. 1997b). Also, several enzymes involved in isoprenoid biosynthesis have been localized to the plastids (Bouvier et al. 1998; Wildermuth and Fall 1988; Zhu et al. 1997; Hugueney et al. 1996), although in most but not all (Kim et al. 1996) of the species studied, the HMGR enzymes have been detected from membranes of endoplasmic reticulum (Campos and Boronat 1995; Enjuto et al. 1994). Whether all the enzymes responsible for mevalonate and GAP/pyryvate pathway are encoded by nuclear DNA (Zhu et al. 1997; Maldonado-Mendoza et al. 1997; Blanc and Pichersky 1995) is still unclear but if not, it could facilitate the use of protoplast fusion as a tool for metabolic engineering via cellular compartmentalization.

5.5.4 Alteration of tissue-types synthesizing isoprenoids

Isoprenoids are usually accumulated to specific tissue types such as flower heads in tansy (Dobos et al. 1992; Hendriks et al. 1990) and pyrehrum (Head 1966) or glandular trichomes of leaves in many species (McCaskill and Croteau 1995; Burnett et al. 1993; Zito et al. 1983). The expression of genes encoding the HMGR such as *hmg*2 (Enjuto et al. 1995; Enjuto et al. 1994), *hmg*2.2 and *hmg*3.3 (Korth et al. 1997) in developing flowers (Burnett et al. 1993); and *hmg* (Burnett et al. 1993), *hmg*2 (Enjuto et al. 1995) and *hmg*1.2 (Bhattacharyya et al. 1995) in pollen have been especially high, reinforces the important role of mevalonate and HMGR activity during flowering (Bhattacharyya et al. 1995; Enjuto et al. 1995; Burnett et al. 1993). Because somatic hybrids have often larger flowers (Matsumoto 1991) or leaves (Rambaud et al. 1992), flowers are often sterile (Rambaud et al. 1997; Trabace et al. 1996; Krasnyanski and Menczel 1995; Chupeau et al. 1994; Rambaud and Vasseur 1994; Matsumoto 1991), flower colour has been altered (Al-Atabee et al. 1990) or the flowering occurs earlier than in the parental species (Trabace et al. 1996; Krasnyanski and Menczel 1995), it is possible to manipulate tissues important for isoprenoid formation. However, a very limited number of studies is available concerning the alteration of isoprenoid composition by the modification of isoprenoid synthetizing tissues through protoplast fusion.

5.5.5 Alteration of metabolic flux within isoprenoid pathway

In a true somatic hybridization the genome is derived from both of the parental species, the process which can alter also the chemical composition of the hybrid. Because a wide variation exists in the capacity of tissues to produce different compounds, it is important to select the donor material from the best clones or tissues. In pyrethrum disc-flowers (Barthomeuf et al. 1996), receptaculums (Zieg et al. 1983), and leaves (Dhar and Pal 1992; Sarker and Pal 1991) have been good sources for *in vitro* explants for pyrethrins production. An interesting possibility to develop high yielding 'plant hybridomas' from two high yielding parental tissues, was mentioned to be a potential advantage of protoplast fusion technique (Yamada and Morikawa 1985). A somatic hybrid between peppermint (*Mentha piperita* L.) and gingermint (*Mentha gentilis* L.) resulted in the formation of shoots with volatile oil constituents and flavor were intermediate compared to the parental plants (Sato et al. 1996; Ishikawa and Sato 1994). The major compounds of the *in vitro* grown somatic hybrids were menthone, menthol and linalool, among the former two were the major volatile components of peppermint and of the latter gingermint (Sato et al. 1996). Protoplast fusion between peppermint (*Mentha piperita* L) and spearmint (*Mentha spicata* L; resistance to *Verticillium* wilt) resulted in somatic hybrids of which most were less resistance to *Verticillium* wilt than spearmint but had the essential oil composition typical to spearmint (Krasnyanski et al. 1998).

Steroidal glycoalkaloid agycones (SGAA) were analyzed from leaves and tubers of somatic hybrids between *Solanum tuberosum* x *Solanum brevidens* (Laurila et al. 1998) and *S. tuberosum* x *S. circaeifolium* subsp. *circaeifolium* Bitter (Mattheij et al. 1992). In addition to the parental type of SGAAs such as solanidine and solanthrene in *S. tuberosum* and tomatidine in *S. brevidens*, somatic hybrids contained also a novel SGAA, demissine. The content of demissine in the hybrid leaves correlated positively with the genome doses of *S. brevidens* (Laurila et al. 1996). Also demissine, as a novel SGAA, was identified from the somatic hybrids of *S. tuberosum* x *S. circaeifolium* (Mattheij et al. 1992). Protoplast fusion between potato and tomato resulted in hybrids, which derived their solanine and chaconine from potato and tomatine glycoalcaloids from tomato. The total glycoalcaloid (GLA) content was higher in the elongated tubers but lower in the leaves of the hybrids compared to the tubers and leaves in the parental tissues, respectively (Roddick and Melchers 1985). In most cases the chemical composition of the somatic hybrids between *Nicotiana tabacum* and *Nicotiana debneyi* were intermediate between the two parental species. The concentration of solanesol was either higher or intermediate compared to the parental species whereas the other terpenoid compounds (cembranes, and plastid pigments: chlorophyll a and b, neoxanthin, violxanthin, lutein, ß-catotene) were either the same or intermediate compared to the parental species (Court et al. 1992).

Possible route for metabolic engineering via protoplast fusion is to alter the metabolic flux within isoprenoid pathway. It was observed that the inhibition of carotenoid biosynthesis resulted in elevated GA levels and reduced ABA content, the compounds which all are derived from isoprenoid pathway. Also changes in sterol and ß-amyrin (triterpene) accumulation was detected in the tomato pericarp due to inhibited carotenoid synthesis during fruit development (Fraser et al. 1995). As a result of protoplast fusion the hybrids were frequently albino or chlorophyll deficient (Wang and Binding 1993), the deficiency which is associated with the segregation of chloroplasts, one possible site for isoprenoid formation (Heintze et al. 1994). In pyrethrum, cryopreservation (-196°C) of flower head

derived callus for 30 days caused the decrease in chlorophyll content, but increased the concentration of pyrethrins I and II, presumably due to the increased flux of IPP into pyrethrins instead of chlorophyll (Hitmi et al. 1997). On the other hand also increased amount of chlorophyll, especially in the stomatal cells has been observed in somatic hybrids (Rambaud et al. 1992). As stated earlier, reports concerning the isoprenoid composition of a somatic hybrid having an altered chlorophyll or other isoprenoid composition are very limited.

II GOALS OF THE STUDY

In this thesis tansy (*Tanacetum vulgare* L.), a wild and underexploited plant species adapted to Nordic climate and characterized by its repellent bioacitivity particularly against insects was the species of interest. The constraint related to the bioactive compounds of tansy, isoprenoids, for further utilization is that they exist at a low concentration in the plant and they are not as active against insects as related isoprenoids, pyrethrins, derived from a related species pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.). However, pyrethrum does not tolerate the long and cold winters of Northern Europe. Pyrethrins are environmentally benign compounds, the production of which does not supply the current and predicted demand in the world market. A new crop, adapted to Arctic growing conditions and producing valuable secondary metabolites, the accumulation of which may be further positively altered in the Northern environment would have a significant impact for Finnish plant production.

The first aim of this thesis was to detect chemical and genetic variation of the selected Finnish tansy lines and to correlate the variation to morphology and geographical origin. Chemical biodiversity of tansy was also studied to detect whether there are chemotypes accumulating irregular monoterpenes which are biochemically closely related to the synthesis of pyrethrins. Theoretically, the use of donor tissue from tansy and pyrethrum which both produce the same irregular monoterpene, chrysanthemic acid, would facilitate the production of a somatic hybrid with increased monoterpene accumulation, the precursor for pyrethrins. The second aim was to establish micropropagation protocol for tansy and pyrethrum and to optimize the culture conditions for genotypes used as tissue donors for protoplast isolation experiments. The regeneration ability of tansy explants for the assessment of preliminary regeneration conditions for protoplasts was also studied. The third aim was to develop a protoplast isolation method for tansy and pyrethrum and to detect the ability of protoplast-derived callus to regenerate. The fourth aim was to develop a protoplast fusion method between tansy and pyrethrum, to progress towards regeneration and to select and apply methods to test the hybridity of fusion products. Protoplast fusion method was chosen as a tool because preliminary experiments to cross-pollinate tansy and pyrethrum were not successful.

The specific objectives were:

1) To detect genetic variation of tansy by RAPD-PCR and by nuclear DNA content, and to identify volatile compounds extracted from flower heads with GC-MS. Selected morphological characters and the origin of the accessions were used to further describe biodiversity in Finnish tansy.

2) To develop *in vitro* culture methods for tansy and pyrethrum which are suitable for micropropagation and for production of donor tissue used for protoplast isolation.

3) To develop protoplast isolation method for tansy and pyrethrum and a chemical fusion procedure between tansy and pyrethrum; and to test the hybridity of fusion products by RAPD-PCR, nuclear DNA content and by analyzing the volatile compounds with GC-MS.

III MATERIALS AND METHODS

The following chapter is structured according to the themes of the original publications (I-VI), including biodiversity of tansy (I, II), establishment of shoot tip culture for tansy and pyrethrum (III, IV, V), callus formation and regeneration from *in vitro* explants from tansy (IV, V), and establishment of protoplast technology for tansy and pyrethrum (IV, V, VI).

1 Plant material (I, II, III, IV, V, VI)

Twenty tansy genotypes (*Tv* 1-20) were collected from different locations in Finland and transplanted to the orchard of the Department of Plant Production, University of Helsinki in 1991 as described in paper I. The explants or other plant material used of tansy for *in vitro* cultures (III, IV, V and VI) or for the detection of volatile compounds (II) were excised from the plants grown in the orchard. Root stocks of all of the tansy genotypes were transplanted also in the greenhouse and shoot tips were transferred to *in vitro* culture. The determination of nuclear DNA content (I) and the extraction of total cellular DNA (I) were carried out on plant material from these plants. Only in one case seeds of a wild tansy accession (*Tv* 50) were sown in the greenhouse and the plant material was used for *in vitro* experiments as described in papers V and VI.

Pyrethrum seeds were obtained from the Pyrethrum Experimental Station Molo, Kenya and sown to pots in the greenhouse as described in paper V.

2 Methods used in *in vitro* culture

2.1 Eradictation of endophytic bacteria from tissue culture (III)

2.1.1 Isolation and characterization of bacteria

Explants of tansy genotypes were cultured on MS medium (Murashige and Skoog 1962) supplemented with a low concentration of NAA (1.07 µM). Growth of bacteria became visible in 5 months after explants had been established in *in vitro*. Samples of bacteria were taken from the immediate vicinity of the roots of symptomless *in vitro* plantlets. Bacteria were spread onto tryptic soy agar (TSA) and pure cultures were obtained through repeated cultures of a single colony at +28°C on TSA. Cultural characteristics were determined and biochemical tests were carried out.

2.1.2 Sensitivity of bacteria to antibiotics

Five antibiotics were evaluated at four concentrations. The antibiotics were: rifampicin (Sigma); the aminoglycosides streptomycin sulphate (Sigma) and gentamicin sulphate (Sigma); and the ß-lactams cefotaxime sodium salt (Claforan, Hoechst) and ampicillin (Sigma). Bacteria inocula for the tests were prepared from fresh cultures grown on nutrient agar (NA; Oxoid) and adjusted to a density of 10^7 cells ml^{-1}. For each bacterium and antibiotic, four replicate tubes at each antibiotic concentration were inoculated for 3 days.

A sample of each tube was transferred on to NA plates which contained no antibiotics and the growth of bacteria was assessed.

2.1.3 Sensitivity of *in vitro* shoot cultures to antibiotics

Sensitivity of shoot cultures to several concentrations of cefotaxime, gentamicin, rifampicin, and to combinations of gentamicin and cefotaxime, and gentamicin and rifampicin was tested using three, contaminant-free axenic tansy genotypes. Four shoot tips of equal size were transplanted into Magenta-7 (Sigma) tissue culture vessels containing MS medium supplemented with 2.66 µM BAP and the antibiotic being tested. The 0-control contained BAP but no antibiotics, whereas the 00-control contained neither BAP nor antibiotics.

2.2 Micropropagation (I, III, IV, V, VI)

Surface sterilization of shoot tips of tansy (*Tv*1- 20, and 50) and pyrethrum (*Tc* 18, 21, 22, and 24) (I, III, IV, V, VI), embryos (*Tv*142094), (IV, V), seeds of pyrethrum (*Tc* 30, 31, 32, and 95) (V), media preparations as well as tissue culture methods were carried out as described in the original papers. For micropropagation MS medium supplemented with sucrose (30 mg l^{-1}), agar (6-6.3 g l^{-1}) or phytagel (2 g l^{-1}), NAA (0 - 10.74 µM), and BAP (0 - 8.88 µM) (pH 5.8) (IV, V) was used. Occasionally, antibiotics (III) or silver thiosulphate (2 mg l^{-1}) (V) were added to the culture media. Seeds and embryos were germinated in the dark at 24±1°C (IV). Plantlets for micropropagation were grown under a 16 h photoperiod with illumination from fluorescent lamps (23±2 - 25±2 °C /16±2 - 20±2°C) at 40 - 150 µmol m^{-2} s^{-1}.

2.3 Shoot tip culture for protoplast isolation (IV, V, VI)

For protoplast isolation tansy and pyrethrum clones were cultured on MS medium supplemented with sucrose (30 g l^{-1}), agar (6 g l^{-1}) or phytagel (2 g l^{-1}), with or without NAA (1.6 µM) and silver thiosulphate (2 mg l^{-1}) under a 16 h photoperiod with illumination from fluorescent lamps (24-25 ±2°C/16-20 ±2°C) at 20 - 200 µM m^{-2} s^{-1}. Shoot cultures were transferred to low illumination prior to protoplast isolation or they were continuously cultured under a low illumination (20 - 40 µM m^{-2} s^{-1}).

2.4 Formation of callus and suspension cultures from leaf explants (V)

Callus was established from small pieces of leaf explants of tansy cultured on MS medium supplemented with NAA (24.17 µM), BAP (19.98 µM), sucrose (30 g l^{-1}), and agar (6 g l^{-1}) (pH 5.8). After 6 months small pieces of callus were transferred to an Erlenmeyer flask filled with liquid MS medium supplemented with CH (500 mg l^{-1}) with or without 2,4-D (4.52 µM) on a rotary shaker.

2.5 Regeneration of shoots from petiole and leaf explants (IV)

Assessment of the efficiency of callus growth and subsequent shoot regeneration from petiole and leaf explants of tansy was carried out to establish a guideline for media components for protoplast culture. Leaf and petiole explants excised from tansy grown *in vitro* were cultured on MS medium supplemented with sucrose (30 mg l^{-1}), phytagel (2 g l^{-1}) with or without casein enzymatic hydrolysate (CH) (500 mg l^{-1}), and different concentrations of NAA (0 - 24.17μM) and BAP (0 - 19.98 μM). Additionally, explants were grown first either in the dark for four weeks and transferred subsequently to the light or under a constant 16 h photoperiod (20±1°C/16±1°C).

3 Protoplast technique

3.1 Isolation of protoplasts (IV, V, VI)

About 100 - 500 mg of fully expanded leaves of *in vitro*-grown plantlets were excised, cut into strips and preplasmolyzed in W5 solution (Menczel et al. 1981). Occasionally excised leaves were preconditioned on agar (6 g l^{-1}) solidified MS medium supplemented with various concentrations of NAA and BAP for 2 days prior to protoplast isolation. Alternatively, protoplasts were isolated from 1 - 1.5 g of callus from the suspension culture (V). The enzyme solution occasionally supplemented with ascorbic acid was incubated at 23 - 30°C with or without shaking for 16 - 22 h. The concentrations and composition of enzymes for protoplast isolation from tansy and pyrethrum were as described in papers IV, V, IV. Digested leaf material was filtered through a sieve and spun at 70 x g for 6-7 min to float or pellet the protoplasts depending on weather sucrose or mannitol was used in the enzyme solution. The washing of protoplasts was carried out as described in papers IV and V. The density of washed protoplasts was determined with a haemocytometer and the viability was tested using FDA.

3.2 Protoplast fusion (VI)

Washed protoplasts of *in vitro*-grown tansy genotypes (*Tv* 142094 and *Tv* 50) were mixed equally (1:1) and resuspended with W5 solution to a concentration of 2 x 10^6 protoplasts ml^{-1}. Leaf protoplasts of *in vitro*-grown pyrethrum (*Tc* 22) were adjusted to the same concentration. In the fusions tansy was fused either with another tansy genotype (F43) or with pyrethrum (F46). The fusion solution contained PEG (15%), $CaCl_2 \cdot 2H_20$ (60 mM), mannitol (90 mM), glycine (25 mM), and DMSO (10%). The fusion procedure is described in paper VI. After the fusion, the density of protoplasts was determined with a haemocytometer and the viability tested using FDA.

3.3 Protoplast culture (IV, V, VI)

Purified protoplasts, either fused or non-fused, were resuspended at a density of 5 x 10^4 - 5 x 10^5 cells ml^{-1} in modified and liquid MS media the composition of which is described in papers IV, V, and VI. To maintain protoplast viability, experiments with three different antibiotics (gentamicin, cefotaxime, and rifampicin) added in four different concentrations

to the culture medium were carried out (V). Tansy protoplasts (1.5 ml) (with or without antibiotics) were cultured in 5-cm-diameter Petri dishes and the cultures were refreshed 4, 7, and 10 days after protoplast isolation by media described in papers IV and V. About 14 days after protoplast isolation the cultures were solidified and thereafter the liquid media surrounding the solid culture refreshed every 7^{th} day. The concentration of salts was gradually changed during the 1-1.5 months of culture to correspond to the concentration of salts in regular MS medium. Simultaneously, the concentration of glucose was decreased and replaced by sucrose. Protoplasts were cultured in the dark at 29±1°C until callus colonies were visible.

3.4 Culture of protoplasts-derived calli (IV, V, VI)

Protoplast-derived calli of tansy (1-2 mm) were transferred to MS medium supplemented with sucrose and different growth regulators (auxins, NAA, 2,4-D; cytokins, BAP, IBA, IPA, kinetin; gibberellic acid, GA3) (IV). In another experiment different concentrations of sugars (glucose, mannitol, or sucrose; 10, 20 or 30 g l^{-1}), combinations of auxins (NAA, 2,4-D, IAA) and cytokinins (BAP, IBA, kinetin, Zeatin), and silvernitrate were tested to achieve cell differentiation (V). The calli were cultured under a 16 h photoperiod in 24-25±2 °C /18-20±2°C at 40-200 µM m^{-2} s^{-1} from fluorescent lamps and subcultured every 20 - 30 days.

3.5 Sensitivity of protoplast-derived calli to antibiotics (V)

To study the potential of reducing the risk of internal bacterial contamination on callus viability of tansy and the possible inducing effect on organogenesis, homogenous calli from *Tv* 142094 protoplast were transferred to MS medium supplemented with NAA (4.30 µM), BAP (28.42 µM) and the antibiotics tested. These were gentamicin, cefotaxime, and rifampicin and the combination of gentamicin + cefotaxime, and gentamicin + rifampicin at several concentrations.

4 Analyses methods

4.1 Determination of nuclear DNA content (I, VI)

Nuclear DNA content (2C value) was determined by flow cytometry in 13 *in vitro*-grown tansy genotypes which had been in tissue culture for 6-8 months. Nuclei were isolated from 30 mg of fully opened top leaves and analyzed as described in paper I. Barley nuclei (cv 'Sultan') were added to tansy nuclei as an internal standard. Relative fluorescence of the propidium iodide-stained nuclei was measured with an EPICS Profile flow cytometer at the Flow Cytometry and Image Facility, Biotechnology Center, Cornell University (I).

Nuclear DNA content (2C value) was determined by flow cytometry also from fusion derived calli (F43A, B, C, and F46A, B), protoplast-derievd calli of tansy (*Tv* 142094; *Tv* 93), leaf tissues of *in vitro*-cultured tansy (*Tv* 142094, *Tv* 50) and pyrethrum (*Tv* 18, *Tv* 21, *Tv* 22, and *Tv* 24). The solution and sample preparations were carried as described in paper VI. Relative fluorescence of the propidium iodide-stained nuclei was measured with

Coulter Epics Elite flow cytometer at the Cytometry Laboratories, Department of Basic Medical Sciences, Purdue University (VI).

4.2 Extraction of total cellular DNA (I, VI)

Total cellular DNA was extracted from 20 greenhouse-grown tansy genotypes (*Tv* 1-20). Leaves were taken from each genotype before flowering, washed with distilled water, and frozen in liquid nitrogen until DNA extraction. The methods used for DNA extraction, purification of DNA using a $CsCl_2$-gradient, and the determination of DNA concentration were carried out as described in paper I.

Total cellular DNA was extracted also from frozen fusion derived calli numbers F43A, F46B, and F46C, from leaves of *in vitro*-grown tansy genotypes *Tv* 142094 and *Tv* 50, and *in vitro*-grown pyrethrum line *Tc* 22. Small scale DNA extraction and DNA concentration determination were carried out as described in paper VI.

4.3 RAPD-PCR analyses (I, VI)

The polymerase chain reaction of DNA extracted from 20 tansy greenhouse grown genotypes was carried out using 10-mer random primers. The sample preparations for RAPD-PCRs, the RAPD-PCR reactions, and the runs of the samples were carried out as described in paper I .

The PCR reaction was also performed on the six *in vitro* grown samples of the fusion experiments (F43A, F46B, F46C, *Tv* 142094, *Tv* 50, *Tc* 22) and from artificially mixed (1:1) sample of *Tc* 22 with *Tv* 142094 or *Tv* 50 using four random 10-mer primers. The sample preparation for RAPD-PCRs, the RAPD-PCR reactions, and the runs of the samples were carried out as described in paper VI.

4.4 Extraction of volatile compounds for GC-MS analyses (II, VI)

Flower heads of 20 tansy genotypes (*Tv* 1-20) grown in the orchard were excised at the onset of flowering, air dried at 38°C, and stored in room temperature in the dark until extracted. Two grams of crushed flower heads were extracted with methanol and petroleum-ether, dried with anhydrous sodium sulphate, stored and evaporated as described in paper II. The extraction of flower heads of each tansy genotype was carried out four times.

Leaves of tissue cultured tansy (*Tv* 142094), leaves of greenhouse grown pyrethrum (*Tc* 22), protoplast-derived callus of tansy (*Tv* 142094), and two fusion-derived callus clones (F43A and F46B) were extracted with dichloromethane as described in paper IV.

4.5 Gas chomatography - Mass spectrometry analyses (II, VI)

Prior to the gas chromatography (GC) analysis, an internal standard was added to three extractions of the 20 tansy genotypes, and the fourth was run without the standard. A sample of 1 µl was injected into the gas chromatograph fitted with a flame-ionization detector. The conditions and programs used for GC and thereafter for GC/MS analysis to identify the compounds are described in paper II.

Samples of 1 µl of the extractions of the fusion experiments (greenhouse grown *Tc* 22, tissue cultured *Tv*142094, protoplast-derived callus *Tv*142094, fusion callus F43A and F46B) were injected and electron impact mass spectra were obtained on gas chromatograph linked to mass selective detector with fused silica capillary column. The detailed conditions used are described in paper VI.

4.6 Morphology and date of flowering (I, II)

Selected morphological characteristics of the 20 tansy genotypes (*Tv* 1-20) grown in the orchard were observed and recorded for 3 years (1993-1995) at the beginning of flowering. Height of the shoot and corymb, the number of nodes per stem, and the number of flower heads per stem were measured from ten shoots per genotype in each year. Observations on the date of flowering were also made for 3 years (1994-1996).

5 Statistical analyses

5.1 Sensitivity of *in vitro* shoot cultures to antibiotics (III)

The data on sensitivity of the *in vitro* shoots to antibiotics were transformed to logaritmic or square root values as appropriate to achieve homogenity of variances. Analysis of variance was performed using the SAS ANOVA procedure and significant effects ($P<0.05$) were included in a regression model with regression computed via the SAS REG procedure.

5.2 Effect of culture conditions on the yield of protoplasts and the growth of protoplast-derived calli (V)

The protoplast yield data was transformed to logaritmic values to homogenize variances The effect of culture conditions on the protoplast yield and the effect of auxins and cytokinins on the growth of protoplast-derieved calli was tested with SAS MIXED procedure. The pair-wise comparisons between culture conditions and between different auxin-cytokinin combinations were done with Tukey's mean separation test.

The effect of antibiotic treatments on the growth of protoplast-derived calli were analyzed with analysis of variance using the SAS ANOVA procedure. Significant effects ($P<0.05$) were included in a regression model with regression computed via the SAS REG procedure.

5.3 Genetic variation (I, VI)

The results on nuclear DNA content of the flow cytometry analysis were analyzed using the SAS GLM procedure of General Linear Model for Variance Analysis and Student-Neuman-Keuls (S-N-K) test as described in paper I.

Reproducible PCR bands were scored on the basis of the presence or absence of a fragment. The SAS program modified by Levy et al. (1991) was used to analyze the distance matrices from RAPD-PCR profiles and to create dendrogram from distance matrices. The dendrogram was synthesized by complete linkage cluster analysis as described in papers I and VI.

5.4 Morphological variation (I, II)

Morphological data were calculated first using the SAS GLM procedure of General Linear Model for Variance Analysis and Student-Neuman-Keuls (S-N-K) test as described in paper I. Thereafter the morphological data were rearranged according to the grouping of principal components of the volatile compounds of tansy. To homogenize variances, the observations of plant height, number of nodes and number of flower heads were transformed to logarithmic values. The differences in morphology between the groups were calculated with SAS CONTRAST program as described in paper II.

5.5 Variation in volatile compounds (II)

The extraction components of the 20 tansy flower heads were analyzed with two different methods. In the first, compounds were scored as being present (I) or absent (0) in an extracted oil. The pair-wise distance matrices of volatile compounds were calculated using the SAS program modified by Levy et al. (1991), similarly as we reported using RAPD-data. In the second method the SAS PRINCOMP program was used to calculate principal components for each of the genotypes. The principal components were created using the concentration of the 55 identified compounds.

5.6 Comparison between data on RAPD-PCR and morphology or volatile compounds (I, II)

To compare the RAPD data with the morphological data, principal components of both types of data with the SAS PRINCOMP procedure were synthesized and plotted to a scatter diagram. The distance of principal components between groups were pair-wise tested using the t-test of SAS as described in paper I. Pearson’s correlation coefficient between the pair-wise distance matrices calculated from the presence or absence of a RAPD-PCR pattern and a volatile compound of the 20 tansy genotypes was calculated with SAS as described in paper II.

IV RESULTS

The following chapter is structured according to the themes of the original publications (I-VI) as indicated in the chapter Materials and Methods.

1 Biodiversity of tansy

1.1 Morphological variation (I, II)

The mean height of tansy genotypes varied from 60.5 cm (*Tv* 19) to 115.4 cm (*Tv* 15), the mean number of nodes from 14.6 (*Tv* 14) to 26.7 (*Tv* 19), the mean number of flower heads per stem from 17.6 (*Tv* 19) to 79.8 (*Tv* 16), and the mean length of the corymb from 4.9 cm (*Tv* 19) to 24.8 cm (*Tv* 16). The date of onset of flowering varied from the 20th of July (*Tv* 3 and *Tv* 15) to the 4th of August (*Tv* 12). Significant differences were found among the tansy genotypes in all of these traits (P=0.0001; for the date of flowering P=0.05). Pearson correlation coefficients between genotype's height and number of flower heads, and height of corymbs were 0.53, and 0.48 (P=0.0001), respectively. Pearson correlation coefficient between the number of flower heads and height of corymb, and date of beginning of flowering were 0.78 (P=0.0001), and 0.35 (P=0.1), respectively.

Principal components 1 and 2 of tansy genotypes based on the morphological data are illustrated in paper I, Fig. 2. Out of the 9 genotypes belonging to group I, 6 were positioned below the principal component 1-axis, whereas 8 out of the 11 genotypes of group II were above the principal component 1-axis.

1.2 Genetic variation (I)

The overall mean 2 C value of nuclear DNA content for tansy genotypes was 8.86 pg. The means of the different genotypes ranged from 7.84 pg (*Tv* 14) to 9.95 pg (*Tv* 4) (I, Table 4) and differed significantly (P=0.05).

The genetic distance of the tansy genotypes was analysed by RAPD-PCR. The smallest genetic distance observed was 0.153, between the genotypes *Tv* 18 and *Tv* 19, whereas the widest genetic distance, 0.476, was observed between the genotypes *Tv* 8 and *Tv* 16 (I, Table 5). The mean genetic distance was 0.294 among all of the 20 tansy genotypes included in this study.

Complete linkage cluster analysis initially separated the 20 genotypes to two groups (group I and II) that were further divided into six smaller sub-groups (I, Fig. 5). The widest and shortest normalised maximum distances were 1.62 (1.62 x 0.294=0.476) and 0.52 (0.52 x 0.294 = 0.153) between group I and II, and between genotypes *Tv* 18 and *Tv* 19, respectively.

The first and second principal components are illustrated in Fig. 6 (I), where most of the genotypes that belonged to group I and group II were positioned below and above the principal component -1 axis, respectively (I, Fig. 6).

Most genotypes in the same subgroup based on complete linkage analysis of RAPD patterns (I, Fig. 5) originated from close by geographical locations (I, Fig. 1). Seven out of the 9 genotypes of group I and 10 out of the 11 genotypes of group II originated south and north of latitude 60°3'N, respectively (I, Table 1).

According to the S-N-K test, the mean number of nodes per stem, the mean number of flowers, the mean length of the corymb, and the date of flowering differed significantly between groups I and II (P=0.05) based on the data from RAPD patterns (II, Table 3).

To explore the potential association of RAPDs and moprhological data the following two calculations were carried out. Firstly, the means of principal components 1 and 2 of the genotypes based on RAPD patterns and belonging to group I and II, respectively, were calculated and plotted to a scatter diagram (I, Fig. 3). The data based on genetic differences positioned the groups nearly symmetrically to the origo: group I below the principal-1 axis, whereas group II was positioned above the principal-1 axis. The means of the principal components 1, 2, 3, and 4 differed significantly between groups I and II (P=0.05), which means that the position of the groups differed also significantly from each other. Distant position of the groups based on RAPD profiles indicated genetic differences. Secondly, the means of principal components 1 and 2 of the morphological data of tansy genotypes belonging to group I and II, respectively, were calculated and plotted on a scatter diagram. Similar to the data from RAPD patterns, the position of the groups was nearly symmetrical to the origo (I, Fig. 3). The t-test indicated that the means of the principal components 1, 2, 3, and 4 for group I and II differed significantly from each other which means that the position of the groups differed. This suggests that morphologically these two groups may be different. The position of groups (I and II) based on RAPD and morphological data on the same scatter diagram was distinct. The principal coordinates for group I based on RAPD and moprhological data were positioned opposite in relation to the origo as compared to the position for group II based on RAPD and morphological data.

1.3 Variation in volatile compounds (I, II)

A total of 55 aromatic volatile compounds were detected from the petroleum-ether extraction of dried flower heads of tansy. The concentrations of 47 of the 55 compounds detected varied highly significantly between the tansy genotypes (P<0.0001) and only the genotypic variation in artemiseole, *trans* sabinen hydrate, nerol, nerolidol, spathulenol, caryophyllene oxide and two unidentified compounds was not significant (II, Table 1).

Fifteen of the genotypes had a main chemical component, which consisted of at least 40% of the total peak area of the volatile compounds, and five genotypes contained at least two terpenes as main components. The two first principal components clustered the 20 genotypes into eight groups when plotted in the scatter diagram. Four of the groups contained more than 19.0% of camphor, and the other four groups less than 4.2% of camphor. The pure camphor chemotypes (>65.2%) formed the largest group. In two groups, camphor was detected in significant concentrations (35.7-50.8%) but in association with other compounds, the concentration of which exceeded 5% such as α-pinene, camphene, 1,8-cineole, borneol, pinocamphone, chrysanthenyl acetate, bornyl acetate, isobornyl acetate, and germagrene D. A group containing a high concentration of 1,8-cineole (47.1%) and a lower concentration of camphor (29.4%) was also identified.

Four other groups consisted of genotypes which contained either artemisia ketone, ß-thujone, davanone, or tricyclene + myrcene as a major compound with or without artemisyl acetate and umbellulone (II; Table 1, Figs 1 and 2).

On the basis of absence or presence of a compound, complete linkage cluster analysis divided the genotypes into two groups. These were further divided into smaller groups which differed from each other more than on average tansy genotypes in this study (II, Fig.3). Some similarities on the grouping of the genotypes in the two analysis methods used could be observed. The two most distant groups (*Tv* 7, 8, 10 & 20 and *Tv* 14 & 16) based on the analysis of the absence or presence of the compounds were also distinguished from the other chemotypes in the principal component analysis (PCA). This was especially clear when principal components 1 and 2 based on the concentration of volatile compounds were plotted in a scatter-gram. In addition, the four small groups formed by the complete linkage cluster analysis (*Tv* 14 & 16; *Tv* 9 & 13; *Tv* 4 & 18; *Tv* 8 & 19) (II, Fig. 3) positioned close to each other also in the PCA (II, Fig. 1).

Six of the seven chemotypes which did not contain camphor as the main component originated from Southern Finland, and only the chemotype containing thujone was collected from Eastern Finland. Eight of the 13 chemotypes with camphor concentration exceeding 18.5% originated from Central Finland and five from Southern Finland. Artemisia ketone was found only from genotypes originating from Southwestern Finland.

The eight groups (II; Fig. 1 and Table 2) resulting from the principal component analysis of volatile compounds, could be joined to two clusters based on camphor concentration. The cluster consisting of groups the camphor concentration of which was between 1.4-4.2% had less nodes per stem, more flower heads and a taller corymb, than the other cluster consisting of groups with camphor concentration more than 19.0%.

Distance matrices of volatile compounds were calculated by the same method as the genetic dictance matrices based on RAPD-PCR patterns. Pair-wise comparisons of the matrices showed that Pearson correlation coefficient, 0.407 ($P<0.0001$), had a 41% analogy between relative genetic and chemical differences between the 20 tansy geno- and chemotypes studies.

The 20 tansy genotypes were arranged according to the groups formed as the result of the principal component analysis of the composition and concentration of volatile compounds. The variation of morphology was compared between the groups using the SAS CONTRAST procedure. The group containing 1,8-cineole had the tallest shoots and differed significantly from the others ($P<0.01$ or $P<0.0001$) whereas the two groups containing mixed chemotypes had the shortest shoots. The group having the shortest shoots had the highest number of nodes in the stems, the number of which differed significantly ($P<0.05$, and $P<0.0001$) from the number of nodes in the other groups. The number of flower heads and the height of the corymb in groups containing davanone or artemisia ketone were the highest, and the results differed significantly ($P<0.05$, number of flower heads; $P<0.01$, height of the corymb) from the other groups. The mixed group containing 1,8-cineole-camphor-chrysanthenyl acetate had the lowest number of flower heads per stem differed significantly ($P<0.05$) from the other groups, except for the group containing thujone. Flowering occurred significantly earlier ($P<0.05$) in the group containing thujone compared to the groups containing davanone or artemisia ketone. The

difference in flowering time was significant ($P<0.05$) between the first and last flowering groups.

2 *In vitro* shoot tip culture for tansy and pyrethrum

2.1 Establishment of shoot cultures *in vitro* (IV)

In vitro cultures were successfully established from shoot tips using MS medium supplemented with low NAA (0.54 - 1.07 µM) and BAP (0.44 - 0.89 µM) concentrations. Strong apical growth of the main shoots under low illumination could be suppressed by increasing the concentration of BAP. Embryos excised from surface sterilised flower heads germinated and developed faster into plantlets compared to plantlet development from seeds.

2.2 Role of BAP in micropropagation of tansy (III)

The effect of BAP (2.66 µM) in the MS medium on micropropagation efficiency in tansy was studied simultaneously with the effect of antibiotics on the growth of *in vitro* cultured tansy (III). The initiation of shoots occurred 6.9 and 8.3 days after subculture for plants grown without and with BAP (2.66 µM), respectively. The number of shoots increased from 1.1 to 7.8 per explant in parallel with an increase in growth rate from 4.3 to 44.8 when BAP was added to the growth medium. Of the three genotypes, only *Tv* 5 produced roots consistently and thus the rooting data from only that genotype could be analysed. Plants growing in MS medium containing BAP initiated root growth significantly later than plants growing without growth regulators. The number of roots and the percentage of roots decreased from 10.1 to 4.0 and from 100% to 29.2%, respectively, when the medium was supplemented with BAP. The final pH in media containing BAP was significantly higher than pH in media without BAP.

2.3 Culture of *in vitro* plantlets for protoplast isolation (V)

Culture conditions had a significant effect on the yield of isolated protoplasts. The growth of tansy plants differed depending on the light intensity during the *in vitro* culture. In high light intensity (80-200 µM m^{-2} s^{-1}) the growth of tansy was slow, the anthocyanin coloured leaves were thick, and only a few shoots were produced even in the BAP containing micropropagation medium. In contrast, leaves of tansy grown in low light intensity (20-40 µM m^{-2} s^{-1}) grew larger and no anthocyanin could be detected. The yield of protoplasts isolated from donor tissue cultured under 20-40 µM m^{-1} s^{-1} was the highest (V; Table 1, condition 5). *Tv* 14 and *Tv* 142094 were the most frequently used genotypes for protoplast isolations due to their adventious shoot growth. Also, medium without BAP and with or without a low concentration of NAA was used for the growth of visually ‘normal’-looking plantlets yielding a high number of protoplasts. The effect of other culture conditions of *in vitro* grown plantlets for the yield of protoplasts is summarised in Table 1 (V).

2.4 Problems of endophytic bacteria in *in vitro* cultures of tansy (III)

Occasional contamination, notably caused by bacteria, became visible around roots of *in vitro* plantlets of tansy about 3-5 months after transferring them to tissue culture. This problem led us to study the possibility of endophytic bacteria, and the eradication of these from *in vitro* cultures with antibiotics, and to observe the possible alterations in the growth of tansy caused by the antibiotics.

2.4.1 Characterization of bacteria

All isolated bacteria were Gram-negative. Five isolates were fermentative, lacked the cytochrome C oxidase activity and belonged to Enterobacteriaceae. Three isolates showed respiratory metabolism and belonged to the fluorescent *Pseudomonas*. Two aerobic isolates belonged to neither of these groups (III; Table 1).

2.4.2 Sensitivity of bacteria to antibiotics

No antibiotic alone was effective against all of the bacteria isolated from *in vitro* cultures of tansy (III; Table 2), but the growth of most, but not all, bacteria was prevented by rifampicin and gentamicin even at their lowest concentrations tested. Combinations of rifampicin and gentamicin at the lowest concentrations in the growth medium prevented the growth of all bacteria. Cefotaxime efficiently inhibited the growth of Enterobacteriaceae. In combination with gentamicin, cefotaxime was effective against all of the bacteria except for no.13. Streptomycin was only modestly effective and ampicillin did not effectively control any strain at the tested concentrations (III; Table 2). Thus, only gentamicin, rifampicin and cefotaxime were further tested for their effects on plant growth.

2.4.3 Sensitivity of *in vitro* shoot cultures to antibiotics

Increased concentrations of gentamicin, cefotaxime, and rifampicin delayed the initiation of shoot growth linearly from 8.3 days to 19.0, 22.1, and 20.5, respectively (III; Fig. 2a). Also, a linear genotype x gentamicin interaction was observed (III; Fig. 2b). When the medium contained the combination of gentamicin + cefotaxime or gentamicin + rifampicin, the shoots started to grow 21.7 days or 19.1 days after subculture, respectively. The number of shoots decreased linearly from 7.8 with increasing concentrations of gentamicin to 2.0, with cefotaxime to 2.2, and with rifampicin to 2.3 (III; Figs 1 and 3). The number of shoots was higher in the combination of gentamicin+rifampicin than in the combination of gentamicin+cefotaxime (III; Fig. 1).

The growth rate of shoots was 44.8 in media supplemented with only BAP. Increased antibiotic concentration decreased the growth rate of shoots (III; Figs 1 and 4). Increase of gentamicin concentration decreased the growth rate from 44.8 to 9.5 (III; Fig. 4), and the reduction was rate-quadratic. Increasing concentrations of cefotaxime and rifampicin decreased the growth rate linearly from 44.8 to 16.1, and to 23.3, respectively. A genotype x gentamicin-quadratic interaction was observed (III; Fig. 4). Gentamicin combined with

rifampicin resulted in growth rate which was significantly higher than the growth rate when a combination of gentamicin and cefotaxime was used (III; Fig. 1). Gentamicin, cefotaxime, and rifampicin reduced the shoot height linearly from 4.7 cm to 1.4 cm, 1.8 cm, and 2.2 cm, respectively (III; Figs 1 and 5). A genotype x cefotaxime-linear interaction was observed. The genotypes exhibited different quadratic responses to differing antibiotic concentrations (III; Fig. 5). Shoots in the medium containing the combination of gentamicin and rifampicin were longer than those in the medium containing gentamicin and cefotaxime (III; Fig. 1).

Of the three genotypes, only *Tv* 5 produced roots consistently in most of the treatments, and therefore only *Tv* 5 could be analysed with analysis of variance. The number of roots per explant and the percentage of roots was 4.0 and 29.2%, respectively, in the medium supplemented with BAP. Increased concentrations of cefotaxime delayed the root initiation linearly from 24.0 days to 32.5 days. Root length had a quadratic response to gentamicin. Increased concentrations of rifampicin increased the percentage of rooted plants from 29.2% to 83.4% (III; Figs 1 and 6). The pairwise comparisons (t-test) of *Tv* 5 vs *Tv* 6 and *Tv* 5 vs *Tv* 142094 showed that the percentage of rooted plants of *Tv* 5 was significantly higher than in *Tv* 6 or *Tv* 142094 when plants were cultured in media containing gentamicin, cefotaxime or rifampicin. The root growth of *Tv* 6 was totally inhibited following treatment with gentamicin (III; Fig. 6).

In a few cases, the morphology of the explants was affected by treatment with antibiotics compared to controls (III; Fig. 1). Leaves were dark green at low concentrations of gentamicin and cefotaxime. Leaves turned curly and sometimes thickened becoming chlorotic at high concentration of gentamicin, cefotaxime, and combinations of gentamicin with cefotaxime or rifampicin. Higher concentrations of rifampicin increased the frequency of hyperhydricity of shoots.

Final pH of the media differed significantly between the tansy genotypes *Tv* 5, *Tv* 6 and *Tv* 142094 after 35 days of culture (III; Fig. 7). Final pH of the media exhibited a quadratic response to concentration of gentamicin and rifampicin, with differences among the genotypes (III; Fig. 7).

The linear regression coefficient of gentamicin differed highly significantly ($P<0.001$) from the linear regression curves of cefotaxime and rifampicin for initiation of shoot growth, number of shoots, and height of shoots as tested by t-test. The linear regression coefficient of cefotaxime and rifampicin differed significantly ($P<0.05$) from each other in the initiation of shoot growth, and shoot number.

3 Callus formation and regeneration from explants of tansy (IV, V)

3.1 Callus and suspension culture (V)

For several months the suspension culture derived from *Tv* 4 leaf callus contained globular structures and very few single cells. Culture medium with NAA and BAP promoted the formation of green and globular cell structures whereas 2,4-D resulted in production of light and small cell aggregates. Gradually the size of the globular structures decreased, the

colour lightened and a suspension culture of small 4-6-cell aggregates was established. Small callus aggregates grown in suspension cultures were used for protoplast isolation.

3.2 Callus formation and regeneration from leaf explants (IV)

Dark green and compact callus was produced from the leaf and stem explants, the latter which produced also shoots. Callus formation, but not shoot or root regeneration was enhanced by the addition of CH to the medium. The first signs of callus formation were visible on explants grown in the dark on high NAA (16.11 - 24 17 μM) and BAP (13.32 - 19.98 μM) concentrations in two weeks. In cultures grown in the light callus was observed one to two weeks later than in the dark grown explants and the strong proliferation of callus growth that was initiated in darkness was visible after several subcultures. Without NAA the callus formation from leaf and petiole explants, and root and shoot regeneration from callus was very poor although the medium was supplemented with different concentrations of BAP. As the medium contained various combinations of both NAA and BAP the morphogenesis was enhanced. The best response was observed with the high concentration of NAA (16.11 - 24 17 μM) whereas the concentration of BAP varied (6.66 - 19.98 μM) in the medium (IV; Table 3). Generally, high concentrations of both NAA and BAP increased the callus formation from the explants in the MS medium.

The best shoot regeneration was obtained on the highest concentration of NAA (24.17 μM) and BAP (19.98 μM) in the MS medium, whereas the highest frequency of root regeneration occurred on the MS medium containing 8.06 μM NAA only (III; Table 3). Most of the regenerated shoots of tansy were hyperhydric. However, also vigorous shoots which had normal appearance and produced roots after transfer to hormone free MS medium were obtained.

4 Protoplast technique for tansy and pyrethrum

4.1 Culture conditions for protoplast isolation (V)

The effects of culture conditions on the yield of protoplasts from tansy and pyrethrum are summarised in paper V, Table 1. Culture conditions had a significant effect on the yield of isolated protoplasts. The yield of protoplasts isolated from donor tissue cultured under 20-40 μM m^{-1} s^{-1} and incubated at 29±1°C in the low enzyme concentration (V; Table 1, condition 5) and supplemented with sucrose (+ a low concentration of $CaCl_2$) was the highest (V; Table 1, condition 5). In tansy, this combination of culturing conditions differed significantly from conditions 1 ($P<0.05$), 2 ($P<0.001$), and 4 ($P<0.05$) and in pyrethrum it differed significantly from conditions 1 ($P<0.001$) and 2 ($P<0.05$) (V; Table 1.). Overall, pyrethrum yielded less protoplasts than tansy. Visually, leaf tissue producing a low yield of protoplasts was usually brown after enzyme incubation. Ascorbic acid added to the enzyme solution decreased the browning but also decreased the yield of protoplasts isolated. Preconditioning the leaves for 2 days on MS medium decreased the browning but did not affect the viability or the protoplast yield. If preconditioned longer, viability and yield decreased.

4.2 Effect of season on protoplast isolation (V)

The percent of successful protoplast isolations and the mean protoplast yields per season are illustrated in paper V, Fig 1. The Pearson correlation coefficient between the mean percent of successful protoplast isolations and the mean yield of protoplasts of the experiments carried out in autumn (September-November), winter (December-February), spring (March-May) or in summer (June-August) was 0.98 for tansy and 0.53 for pyrethrum.

Protoplast isolations performed during the period from December to April yielded the highest number of protoplasts whereas the division of tansy protoplasts and the formation of callus were most successful in February, March, and April. During that time in total 32 successful protoplast isolations from tansy were performed of which 12 isolations from *Tv* 142094 and 3 isolations from *Tv* 50 resulted in callus formation. Up to 1.0% of the isolated protoplasts formed callus and more than 3000 calli per isolation could be obtained. Pyrethrum formed microcolonies which did not develop further.

4.3 Protoplast isolation (IV, V)

In total 488 protoplast isolations were performed of which 309 were from tansy, 158 from pyrethrum, and 15 from the tansy cell suspension culture. Of these isolations 187 (60.9%), 96 (58.5%), and 11 (73.3%) yielded protoplasts from tansy, pyrethrum and tansy cell suspension culture, respectively. The mean yields of the protoplasts isolated from tansy, pyrethrum, and the suspension culture were 6.0 x 10^6, 3.0 x 10^6, and 7.5 x 10^4 g^{-1} FW, respectively. The mean protoplast yield of tansy genotypes ranged from 2.5 x 10^6 (*Tv* 16) to 10.5 x 10^6 (*Tv* 11) g^{-1} FW and among the pyrethrum lines from 1.0 x 10^6 (*Tc* 24) to 5.0 x 10^6 (*Tc* 18) g^{-1} FW. From the 309 protoplast isolations carried out from tansy 232 consisted of genotypes *Tv* 5, 8, 14, or 142094 and from the 158 isolations from pyrethrum, 110 consisted from lines *Tc* 18, 21, or 22 (V).

The diameter of protoplasts ranged between 20-60μm (IV; Fig.1a). Some of the protoplasts contained anthocyanin pigments. The viability of protoplasts increased from 60% up to 95% when relative centrifugal force was reduced from 100 x g to 70 x g during the first spin and to 41 x g during the subsequent three centrifugations during washing of the protoplasts (IV).

4.4 Effect of media components on protoplast culture (IV, V)

Accumulation of brown pigmentation was frequently visible in protoplasts after 4-5 days of culture. However, dilution of the cell mixture decreased the intensity of the pigmentation and improved the growth of the cultures. Later, when protoplasts were plated in agarose, weekly replacement of the surrounding media also hindered the growth reduction by the brown metabolites produced by the cultured cells. Protoplasts grew well and stayed viable in the medium supplemented with 90 g l^{-1} of glucose and a low concentration of ammonium nitrogen as the N-source (IV).

Protoplast viability was usually high (90%->) when a high yield of protoplasts (>5 x 10^6 g^{-1} FW) was obtained. In terms of the antibiotics studied the least reduction in viability was observed during the two week culture period in which rifampicin (12.5-25 mg l^{-1}), gentamicin (25-50 mg l^{-1}), or cefotaxime (100 mg l^{-1}) was added to the medium. At the end of the experiment the viability was the highest when the cells had been cultured in medium supplemented with gentamicin (25 mg l^{-1}) or rifampicin (12.5 mgl^{-1}) (V; Fig. 2) The increase in acidity of the culture medium from pH 5.8 to pH 3.5 - 4 during the first few days after protoplast isolation was prevented by adding 5 mM MES to the medium (V).

The growth hormones NAA and BAP were essential for protoplast division. High concentration of NAA (26.85 μM) promoted cell division with a wide range of zeatin (4.56-45.62 μM) or BAP (4.44 - 44.40 μM). Either symmetrical or asymmetrical protoplast division was observed. Without NAA, BAP or zeatin the protoplasts did not divide, and the division ceased if the protoplast medium was not solidified (V).

4.5 Effect of media components on protoplast-derived callus growth of tansy (IV, V)

In semi-solid agarose (0.35%) loose colonies continued to form microcalli, but in solid agarose (0.6%) the division was very slow or it discontinued (V). Protoplast-derived calli, 1-2 mm in diameter, were transferred from agarose plating medium to fresh medium for further proliferation, usually 2-3 months after isolation (IV; Fig. 1d). Most of the calli reached a diameter of 5-10 mm in 3-4 months after isolation, after which they were transferred to shoot regeneration medium (IV). Protoplast-derived calli grew rapidly in medium containing glucose or sucrose, but grew very slowly and turned brown on growth medium containing mannitol. Silver nitrate (>20 μM) in the growth medium decreased callus growth (V).

4.5.1 Effect of hormone combinations on initial protoplast cultures (IV)

Of the various hormone combinations shown in paper IV, Table 2, modified MS medium (MSIII) with BAP (13.32 μM) + NAA (5.37 μM) resulted in production of green and compact callus with nodules (IV; Fig 1f). BAP (8.88 μM) + GA_3 (0.29 μM) in the MS medium resulted in many green nodules with some specialised arrangements on the surface. Production of anthocyanin was observed also in calli. Different types of callus growth were observed after 3-4 months of culture depending on the combination of hormones as shown in paper IV, Table 2. White, unorganised, and friable callus was induced on media, in which the concentration of auxins exceeded cytokinins. Green, compact, and nodulating callus with meristematic zones was produced on media, in which the concentration of cytokinins exceeded auxins, whereas white, friable and pre-embryogenic calli (IV; Fig. 1e) were observed in association with both of the above mentioned callus types (IV; Table 2). Protoplast-derived calli were subcultured 6-7 times, however, no shoot regeneration occurred.

4.5.2 Further refinement of hormone combinations in protoplast cultures (V)

Calli grown without hormones grew slowly, and no globular or meristematic structures formed. Both auxins and cytokinins had a significant effect on protoplast-derived callus growth. The callus growth was most abundant with NAA (1.07-17.18 μM) and zeatin (0.91-14.60 μM) (V; Tables 2 and 3). Formation of meristematic structures differed significantly only between the auxin treatments. The calli were visually scored 'the most meristematic' when NAA or IBA of the auxins were combined with BAP or zeatin (V; Tables 2 and 4). Formation of meristematic structures was observed especially when IBA (0.98-7.87 μM) or NAA (1.07-8.59 μM) were used in combination with BAP (0.89 - 14.21 μM).

At the end of the 30-day experiment the greenish and most compact protoplast-derived calli were further cultured up to 20 months subculturing every 30-40 days. The calli cultured on MS medium supplemented with BAP (56.84 μM) and/or NAA (68.74 μM) produced green, globular and meristematic formations whereas the calli cultured on high concentrations of BAP (up to 113.68 μM) and/or NAA (up to 137.48 μM) turned brown.

4.6 Sensitivity of protoplast-derived calli of tansy to antibiotics (V)

The increased concentrations of gentamicin decreased the growth rate of calli from 2.21 to 1.15 (V; Fig. 4). When cefotaxime and rifampicin were used alone they did not have an affect on the growth rate. When gentamicin and rifampicin were used in combination, a significant interaction was observed. The increased concentration of rifampicin from 12.5 to 25 mg l^{-1} increased the growth rate of callus from 1.63 to 2.26 ($P<0.05$) at the basal rate of 25 mg l^{-1} of gentamicin. The increased concentration of gentamicin from 25 to 50 mgl^{-1} decreased the growth rate of calli from 2.6 to 1.53 ($P<0.05$) at the rate of 25 mg l^{-1} of rifampicin.

4.7 Regeneration from protoplast-derived callus of tansy (IV, V)

Cell differentiation with root formation was observed on callus cultured on the MS medium with BAP (13.32 μM) + NAA (10.74 μM) supplemented with CH 250 mg l^{-1} (IV; Fig. 1g). Spontaneous shoot formation occurred from protoplast derived callus of tansy cultured on MS medium supplemented with 2,4-D (3.62 μM) and zeatin (1.82 μM) (V; Fig. 3). The growth of the about 5 mm long shoots ended due to contamination. None of the other attempts resulted in shoot formation even if globular structures with trichomes, nodules and anthocyanin pigmentation were frequently observed on media with several hormone concentrations (V).

4.8 Protoplast fusion and characterization of the fusion calli (VI)

In total 48 chemical fusion experiments with PEG between tansy and pyrethrum protoplasts were carried out, of which three resulted in callus formation. The volume of the fusion solution was found critical for the fusion procedure. The optimum volume of the PEG solution was 1.5 x the volume of the protoplasts. If the volume was less (1x) no

fusion occurred, and if it was higher (2x) the protoplasts collapsed. Protoplast viability before and after the fusion was 90-95% and 30-50%, respectively.

4.8.1 Culture of fusion-derived callus

Fused tansy and pyrethrum protoplasts [F46; (*Tv* 50 + *Tv* 142094) + *Tc* 22] began to divide in 2 days, whereas the protoplasts of the intraspecific tansy fusion (F43; *Tv* 50 + *Tv* 142094) started to divide 5 days after protoplast fusion. Two weeks after the fusion small microcolonies containing 30-50 cells were observed in F46, whereas the cells of the F43 had reached only the 5 cell stage. Calli formed from fusion F46 were 1 mm diameter in 20 days after fusion compared to F43 which needed twice that time to reach the same diameter. No divisions were observed in the control plates of tansy and pyrethrum protoplasts.

In total, 5 calli were obtained from the F43 fusion and 12 from the F46 fusion. Calli derived from F43 grew slowly and were friable, unorganised, albino or yellowish, and coloured with anthocyanin. In 6-10 months the first signs of organization such as globular structures were observed. Callus growth was better or similar in MS medium supplemented with 30 or 40 g l^{-1} glucose instead of sucrose. Calli derived from the fusion F46 grew fast and were compact, green and formed globular and meristematic structures. Clear differences in the growth rate and structure between the calli clones derived from the fusion were observed during the 20 months of culture.

4.8.2 Nuclear DNA content

The nuclear DNA content of *Tv* 142094, *Tv* 50, protoplast-derived calli of *Tv* 142094 and pyrethrum were 6.41, 7.39, 8.09-8.15, and 13.16-14.76 pg, respectively, whereas the DNA content of five fusion-derived calli (F43A, B, C and F46A, B) ranged from 8.84 to 31.87 pg (VI; Table 1).

4.8.3 RAPD patters

The DNA yields from fresh calli and leaves were 0.7 - 0.8 μg of total DNA per 100 mg, respectively. Four RAPD primers were used, which produced a total of 56 reproducible bands (350 bp - 2350 bp) (VI; Fig. 1) of which 4 were common for all samples and 52 polymorphic. The genetic distance was the widest (0.714 and 0.762) between pyrethrum and the two tansy genotypes (*Tv* 142094 and *Tv* 50), and the smallest (0.031) between the two callus clones derived from the fusion F46 (F46B vs F46C). Fusion callus F46B [(*Tv* 142094 + *Tv* 50) + *Tc* 22] was similar to *Tv* 50 and to *Tc* 22 (0.472), but the artificial mix of tansy+pyrethrum DNA had more similarity to pyrethrum (distance 0.245 and 0.292) than to the tansy RAPD pattern (distance 0.373 and 0.417). Fusion callus F43A (*Tv* 50 + *Tv* 142094) had a higher similarity to *Tv* 50 (distance 0.269) than to *Tv* 142094 (distance 0.519) (VI; Table 2.). Average linkage cluster analysis separated the samples into two groups. Both of the tansy genotypes and the fusion F43A belonged to one group whereas pyrethrum, fusion F46B, F46C, and artificially mixed DNA from tansy and pyrethrum belonged to the other group (VI; Fig. 2).

4.8.4 Analysis of volatile compounds

Kovat's indices (K_I) (Retention indices, R_I), authentic compounds and mass spectral library were used to identify the composition of the dichloromethane extracts. In total 36 volatile organic compounds (K_I 1800) were quantified, of which 24 were either identified or tentatively identified. No compounds common for fusion-derived calli (F43A, F46B) and tansy (*Tv* 142094) or pyrethrum (*Tc* 22) were observed. Decadienal, artedouglasia oxide, syringaldehyde, heptadecane and coniferyl alcohol were identified only from protoplast-fusion derived calli (F43A, F46B). Coniferyl alcohol was found also from protoplast-derived callus of *Tv* 142094. Beta farnesene and germacrene D were volatiles present in both tansy (*Tv* 142094) and pyrethrum (*Tc* 22) (VI; Table 3).

Among the less volatile compounds (oven temperature 164-260 °C) hexadecanoic and linoleic acid were present in both the protoplast fusion-derived calli (F43A, F46B) and tansy (*Tv* 142094). One unidentified compound (K_I 2264) was detected in the fusion callus (F46B) and the pyrethrum (*Tc* 22). In addition, several compounds were observed only from protoplast fusion-derived calli (F43A, F46B). Four peaks with mass spectra corresponding to the mass spectra of authentic pyrethrins I (K_I 2281 and 2321) and II (K_I 2264 and 2294) were detected from pyrethrum (*Tc* 22) extracts. Peaks with mass spectra corresponding to pyrethrins were not observed in the protoplast fusion-derived calli.

V DISCUSSION

1 Biodiversity of tansy (I, II)

Morphological variation

All the 20 tansy genotypes included in this study differed chemically, genetically, and in the selected morphological characters. Also, linkage of these characters to the geographical origin of tansy genotypes was observed as well as correlation between these three characters. The morphology of tansy genotypes varied, as has been previously reported for Finnish tansy (Hämet-Ahti et al. 1984). Although environment may have a significant effect on phenotype especially of wild species growing as a natural population (Widén 1991), the morphological differences in the tansy genotypes worked on in this thesis remained consistent over the three years of study. The consistency may be due to the permanent production site of tansy genotypes with similar access to sunshine, which has been one of the most determining environmental factors causing morphological variation in wild species (Widén 1991). All the variables examined distinguished at least a few tansy genotypes from the others. The mean number of flower heads per stem was positively correlated with the height of stem and corymb. Because essential oils of tansy are usually concentrated in flowers (Dobos et al. 1992), the selection of genotypes with high flower head number is an important step towards increasing the yield of essential oils. *Senecio interifolius* is another species of Asteraceae that has shown also a positive correlation between the stem height and the number of florets per stem (Widén 1991). Plants of *S. interfolius* with large inflorescences started to flower earlier and had a longer flowering period than plants with smaller inflorescences (Widén 1991). Unfortunately, we could not observe the duration of flowering in our tansy genotypes because the flower heads were collected for distillation of essential oils.

Tansy genotypes from southern and eastern Finland (group I) had longer corymb, and more nodes and flower heads per stem, but they started to flower later than the genotypes native to western and central parts of Finland (group II). This is also consistent with previous observations (Lokki et al. 1973). The division of tansy genotypes to group I and II was significant according to the morphological data subjected to multivariate analysis. Early flowering is an important trait for crop species under Nordic conditions. Therefore, further selection of tansy genotypes will be directed to identification of genotypes, in which the trait of high flower head number and early flowering are combined.

Genetic variation

Genetically tansy genotypes varied in their nuclear DNA content (2C values) and in RAPD-PCR patterns. The nuclear DNA content (2C values) among the 13 genotypes studied varied up to 27%. One explanation for the variation could be the possible occurrence of cytomictic disturbances (Virrankoski and Sorsa 1968). In sexual hybrids of *Heliantus annuus* x *Helianthus tuberosus* chromosome number was not the cause for the alteration in nuclear DNA. In contrast, the variation in hybrids was explained by the variation in the parental species and by the position in the inflorescence where the seeds for the nuclear DNA analysis were collected (Natali et al. 1998). Species in the genus

Helianthus have also shown intraspecific variation in their nuclear DNA content. For example, variation of 9.5% and 15.8-32% have been reported among different *Helianthus tuberosus* (Natali et al. 1998) and *Helianthus annuus* lines (Natali et al. 1998; Michaelson et al. 1991b), respectively, whereas the variation in *H annuus* x *H. tuberosus* hybrid was 21.42% (Natali et al. 1998). The mean 2C value of the 20 tansy genotypes was 8.86 pg, which is higher than most of the 2C values reported for other species of Asteraceae (Arumuganathan and Earle 1991; Michaelson et al. 1991a, 1991b), although nuclear 2C content of 19.91-23.05 pg was reported recently for *Helianthus annuus* genome (Natali et al. 1998).

The analysis of RAPDs showed that all of the 20 tansy genotypes examined could be distinguished. The reason for the detected, relatively high polymorphism, among this species is not clear but it may be contributable to high frequency of cross-pollination among many (Lokki et al. 1973), but not all (Holopainen et al. 1987a), tansy geno- and chemotypes. Previous authors have observed that only less than five percent of the flowers produced seeds after self-pollination (Lokki et al. 1973), and that the expansion of tansy occurs predominately through dispersal of cross-pollinated seeds (Prach 1988; Lokki et al. 1973). However, cross-pollination between certain tansy chemotypes can result in poor seed set which may restrict gene flow and contribute to the different frequencies, in which different tansy chemotypes occur in the nature (Holopainen et al. 1987a). In other species related to *Tanacetum*, such as chrysanthemum (*Dendranthema grandiflora* Tzvelev or *Chrysanthemum morifolium* Ramat.), a strong self-incompatibility mechanism has been observed to prevent gene flow between closely related genotypes and cause variation in polymorphism (Wolf et al. 1995). In contrast, low RAPD polymorphism was found among wild and domesticated sunflower (*Helianthus annuus*) that can produce fertile siblings freely by intraspecific hybridization (Arias and Rieseberg 1995).

RAPD data and morphological data subjected to complete linkage cluster and multivariate analysis grouped the tansy genotypes into two main groups. As the genotypes were arranged to the two groups according to the distance analysis of RAPDs and analyzed for principal components according to their morphology, the grouping was further confirmed. Thus, both the morphological and RAPD data could be used to group the 20 tansy genotypes to similar two main groups, and as explained above, this grouping was related to the geographical origin of the tansy genotypes. In another species in Asteraceae, namely *Silene latifolia* the morphological traits and RAPD patterns showed also correlation with the geographical origin of the genotypes (Vellekoop et al. 1996). Group I of this study included the 7 tansy genotypes from southern Finland that originated south of the latitude 60°3’N, which coincides with the mean annual isotherm of +4 °C (1961 - 1990) (Finnish Meteorological Institute 1997). Interestingly, also the two tansy genotypes *Tv* 2 and *Tv* 4 from the lake district of eastern Finland belonged to group I. Consequently, most of the genotypes of group II originated North of the latitude 60°3’N. These results may be best explained by the different climatic conditions, such as the temperature, south and north from the latitude 60° 3’N and the adaptation of tansy genotypes to the respective growth conditions. *S. latifolia* has been genetically and chemically grouped to western and eastern races, which coincided with the January 0°C isotherm (Vellekoop et al. 1996).

In Finland, the genetic variation between the genotypes originating from different geographical regions may be the result of expansion of agriculture, with the adaptation of tansy to the local climate (I). Tansy has been observed to be one of the most common seed

species in the ballast soil area in Reposaari (Jutila 1996), which has been an important harbor in Southwest Finland. Many plant species have spread to Finland by seed embedded in the ballast soils used in ships (Jutila 1996). The data of this study and previous studies (Hultén 1950; Linkola and Väänänen 1940) suggest that the native area of tansy may be the southern coast of Finland, whereas the tansy genotypes growing in other parts of Finland may have been moved there through agriculture and attempts of domestication.

Variation in volatile compounds

In total, 55 volatile compounds were detected from air dried tansy flower heads of which 53 were identified. The most frequently found compounds exceeding 10% at least in one chemotype were tricyclene, camphene, myrcene, 1,8-cineole, artemisia ketone, ß-thujone, camphor, umbellulone, artemisyl acetate, pinocamphone, myrtenol, chrysanthenyl acetate, bornyl acetate, and davadone, which have been reported in tansy (Collin et al. 1993; Neszmélyi et al. 1992; Hendriks et al. 1990; De Pooter et al. 1989; Gallino 1988; Holopainen 1989; Héthelyi et al. 1981; Ekudayo 1979; Nano et al. 1979; Tétényi et al. 1975; Forsén 1975; Forsén 1974; Forsén and von Schantz 1971; Sorsa et al. 1968; von Rudloff and Underhill 1965). Artemisyl acetate, as a compound exceeding 10% was detected in the present thesis research for the first time from tansy, however, the compound has been identified from related species such as from *Artemisia* spp. (Worku and Rubiolo 1996; Epstein and Gaudioso 1984). Also, an artemisia ketone isomer observed previously in tansy oil (Hendriks et al. 1990) shows a retention index linear with our observation of artemisyl acetate.

In our study, camphor was the most common chemotype, and this is in agreement with others (Hendriks et al. 1990; Holopainen 1989; Ekundayo 1979; Sorsa et al. 1968). In mixed camphor chemotypes, camphene, 1,8-cineole, pinocamphene, chrysanthenyl acetate, bornyl acetate, and isobornyl acetate were the most frequently found satellite compounds (10 - 30%). We also identified three well-defined artemisia ketone chemotypes. Such chemotypes have been observed previously by Forsén and von Schantz (1971) and Sorsa et al. (1968) from Finnish tansy and are common in the Netherlands and Hungary (Hendriks et al. 1990; Tétényi et al. 1975). In contrast to other reports (Holopainen 1989; Forsén 1975; Sorsa et al. 1968) thujone was not among the most common monoterpenes in our study. Thujone was found only in small concentrations except for one chemotype where it was the main component. Our observation was more in accordance with Hendriks et al. (1990), who did not detect high concentrations of thujone together with camphor. The mixed chemotypes containing tricyclene (16 - 27%) and myrcene (21 - 39%) have not been reported previously from tansy in Finland, although Sorsa et al. (1968) found chemotypes containing either ≤-pinene or tricyclene (4 - 14%) accompanied with a low concentration (0.1 - 0.3%) of myrcene. Interestingly, a davanone chemotype, which is reported now for the first time from tansy grown in Finland, has been detected previously only from a Hungarian-grown tansy (Németh et al. 1994; Héthelyi et al. 1991; Héthelyi et al. 1981).

Most minor compounds (concentration <10%) detected here have been reported in tansy, but seven were observed for the first time. All the seven have been detected from related species in Asteraceae family. These include artemiseole from *Artemisia arbuscula*

(Epstein and Gaudioso 1984), isobornyl acetate from *Artemisia vulgaris* (Hwang et al. 1985), nerolidol from *Artemisia lacinata*, and *Tanacetum polycephalum* (Weyerstahl et al. 1997; Rustaiyan et al. 1990, respectively), germacrene alcohol from *Echinacea purpurea* (Bauer et al. 1988), spathulenol from *Achillea millefolium* and *Achilla laciniata* (Afsharypuor et al. 1996a; Weyerstahl et al. 1997), and caryophyllene oxide from *Tanacetum annuum*, *Achillea wilhelmsii* and *Achillea laciniata* (Barrero et al. 1992; Afsharypuor et al. 1996b; Weyerstahl et al. 1997, respectively). These include also artedouglasia oxide which has been detected from *Artemisia laciniata* (Weyerstahl et al. 1997) as well as from protoplast fusion derived callus between tansy x tansy and tansy x pyrethrum (Keskitalo et al. 1999).

Among the 20 tansy genotypes, 15 had a major compound, the concentration of which exceeded 40%, while the oil of five genotypes was composed of several minor compounds. Based upon the definition of `chemotypes` by previous authors (Hendriks et al. 1990; Holopainen 1989), 15 tansy genotypes are 'well-defined' chemotypes, and the remaining 5 are 'mixed' chemotypes. Holopainen (1989) observed that about 20% of tansy chemotypes resulting from their crossing experiments were also mixed chemotypes.

Geographically, most of the chemotypes containing more than 19% camphor originated from Central Finland, whereas chemotypes containing less than 4.2% originated from South or Southwest Finland. This observation is in agreement with Sorsa et al. (1968) who found that camphor was more frequently observed in tansy grown in Northern Finland compared to Southern grown tansy, where thujone was more frequent. Artemisia ketone and davanone were detected only from tansy originating from Southwest Finland. Similar geographical variation in terpene composition and chemotypes of tansy have been observed within and between other countries (Neszmélyi et al. 1992; Hendriks et al. 1990; De Pooter et al. 1989; Gallino 1988; Héthelyi et al. 1981; Ekundayo 1979; Nano et al. 1979; Tétényi et al. 1975; von Rudlof and Underhill 1965). Interestingly, tansy originating from Southwest Finland contained only artemisia ketone or davanone as the main components. These are commonly observed compounds in tansy from Central Europe (Hendriks et al. 1990; Héthelyi et al. 1981). Correlation between the genetic distance and the chemical distance matrices was 41%, showing a analogy between the variation of the two matrices. Unfortunately, only in a few cases has terpene variation been studied simultaneously with genetic variation. In agreement with our study, *Juniperus* species have been successfully differentiated from each other using volatile terpenoid analysis and RAPD patterns (Adams et al. 1993). Wild species of different *Origanum vulgare* populations were observed to vary in the ratio of monoterpene hydrocarbons and phenols in the essential oils due to the altitude of the production site. The elevation of the site from 400-600 meters to >700 meters, increased the phenols/monoterpene ratio from 1.19 to 1.41, respectively, whereas the sum of these compounds stayed relatively unchanged (Russo et al. 1998).

The chemotype 1,8-cineole, had the tallest shoots while the mixed chemotypes were the shortest. Németh et al. (1994) observed that chemotypes containing a thujen-acetate had the tallest shoots whereas plants with 1,8-cineole were the shortest. We also observed that the davadone chemotype had the highest number of flower heads per shoots, which is in agreement with the observation of Németh et al. (1994). Here, the thujone chemotypes produced a low number of flower heads, whereas the thujone chemotype grown in Hungary was among the chemotypes having high number of flower heads. In our study,

the chemotypes containing a high concentration of camphor had less flower heads and initiated flowering earlier than chemotypes low in camphor. This is in agreement with observations by Németh et al. (1994).

The underlying causes of the observed chemical variation in tansy is an intriguing question, and the answer still remains elusive. Genetic variation due to the different geographical origins of tansy (I) may have led to differences in the genetic control of essential oil accumulation (Holopainen 1989; Lokki et al. 1973). A wide variation in essential oil composition presumably has ecological advantages in protecting plants against different pests (Hough-Golstein and Hahn 1992; Neszmélyi et al. 1992; Héthelyi et al. 1991; Nottingham et al. 1991). It is also likely, that part of the terpene expression in tansy is linked to specific environmental or climatic conditions (Sorsa et al. 1968), and to a lesser extent may be an indicator of other characters such as morphology (Németh et al. 1994).

Interaction between characters

The dependency between the geographical origin and genetic, chemical and morphological variation in tansy shows that different factors need to be recognized when biodiversity of herbaceous species is being examined. The analogy between the relative chemical and genetic differences among the 20 tansy chemo- and genotypes, respectively, suggests that different terpene compositions resulting from differential activation of specific enzymes may be related to the variation in RAPD patterns. The association between the main chemical components and morphology should be considered when selecting the parental chemo- and phenotypes from tansy populations for future work. The use of morphological traits as indicators of selected chemotypes would be most useful in breeding and biochemical studies. Since the bioactivity of the essential oil of tansy depends on the composition of terpenes (Héthelyi et al. 1991; Holopainen and Kauppinen 1989; Panasiuk 1984; Schearer 1984), the chemotype with the most effective oil composition should be selected. According to previous studies of bioactivity of tansy oil, artemisia ketone (Héthelyi et al. 1981), camphor (Holopainen and Kauppinen 1989; Schearer 1984), chrysanthenyl acetate (Neszmélyi et al. 1992), 1,8-cineole (Schearer 1984), davanone (Héthelyi et al. 1981), and thujone (Holopainen and Kauppinen 1989) chemotypes are among the most interesting ones.

2 Recalcitrance in i*n vitro* shoot tip culture, callus formation and regeneration from explants (III, IV, V)

Establishment of *in vitro* shoot cultures for micropropagation and as source tissue for protoplast isolation has been developed for tansy using surface sterilized embryos, seeds and shoot tips. In addition, callus formation and regeneration from leaf and petiole explants were studied. Embryos excised from flowers were the most favourable tissues for the establishment of shoot cultures *in vitro* due to rapid germination into plantlets and absence of contamination. However, obtaining plant material of a desirable chemotype from embryos is restricted by the high cross-pollination of *T. vulgare*: less than five percent of seeds have been reported to result from self-pollination (Lokki et al. 1973). Because of the long time required for germination of seeds and the high cross-pollination level of tansy, the most

suitable method to establish *in vitro* culture was from shoot tips. Hyperhydricity, tissue browning, and bacterial contamination, obviously due to internal bacterial colonization, were the most serious problems associated with the *in vitro* cultures of tansy.

Hyperhydricity

Hyperhydricity with curled and brittle leaves was observed in tansy shoot cultures treated with high concentrations of antibiotics. When the phenomenon was analysed by the quadratic regression curve of growth rate; hyperhydricity increased the growth rate and thus, fresh weight of shoots (III). Hyperhydricity was also a problem in regeneration from leaf and petiole explants. About ninety percent of the regenerated shoots of tansy were hyperhydric (IV), the problem which has been frequently observed also in shoots regenerated from protoplasts in the genus *Helianthus* (Trabace et al. 1996; Wingender et al. 1996; Krasnyanski and Menczel 1995; Trabace et al. 1995; Krasnyanski and Menczel 1993; Fischer et al. 1992). In the both above mentioned cases in this thesis, tansy explants were cultured in medium supplemented with relatively high concentrations of BAP. Also other type of cytokinins such as zeatin have caused hyperhydricity (Shibli and Smith 1996). Obviously tansy exhibits sensitivity to BAP and the use of that growth regulator should be taken into account in future studies. However, it may cause a contradictory situation, because BAP was observed to be an important growth regulator for regeneration from explants (IV) and proliferation for protoplast-derived callus (V). Similarly, other explants of plants of the Asteraceae family such as *Chrysanthemum cinerariaefolium* Vis (Pal and Dhar 1984), *Cynara scolymus* L. (Ordas et al. 1991), and *Dendranthema grandiflora* Tzvelev (Kaul et al. 1990) have formed callus only in high cytokinin and auxin concentrations. Because several anatomical (Olmos and Hellin 1998; Jones et al. 1993; Williams and Raji 1991; Capellades et al. 1990; Paques and Boxus 1987) and biochemical (Frank et al. 1995; Gorinova et al. 1993; Le Dily et al. 1993) abnormalities are associated with hyperhydricity, which obviously may have an affect also in regeneration, a question arises, whether the protoplast-derived calli were recalcitrant to regenerate due to hyperhydricity caused by medium supplemented frequently with BAP.

Tissue browning

Browning of leaf tissue became problematic in protoplast isolation experiments of tansy and pyrethrum. Leaf tissue during the enzyme maceration turned brown especially when the donor material was excised from plantlets grown in high light intensity, cultured in a medium supplemented with BAP or if the enzyme solution contained, particularly, mannitol. We overcame the problem by culturing the donor tissue continuously in low light intensity and in a medium without cytokinin, in the environment where plantlets visually observed contained less antocyanin. Several enzymes associated with rapid cell growth and bud formation as a consequence of hormone treatments have been detected to be particularly active in *in vitro* grown *Chrysanthemum morifolium* (Aribaud et al. 1995; Aribaud et al. 1994). According to the authors, the importance of these enzymes is still unclear but they are able to link polyamines covalently to protein substances by an external stimuli or as a consequence of tissue wounding (Aribaud et al. 1995; Aribaud et al. 1994). Some of the amines posses toxic or carcinogenic properties (Luckner 1990) that may have had also a deleterious effect for the cells and/or protoplasts released from sliced and wounded leaves during enzyme incubation. The favourable effect of low light intensity

was also detected for callus growth of tansy explants, and the effect was visible after several subcultures (IV). Also other species in the Asteraceae family had a positive response to low light intensity. Protoplasts isolated from dark grown seedlings of *Helianthus annuus* had a significantly higher division frequency compared to protoplasts isolated from light grown seedlings (Geng-Guang 1996). Also, *Dendranthema grandiflora* produced more somatic embryos when cultured in the dark than explants grown in 16 h photoperiod (May and Trigiano 1991).

Another method to avoid browning of leaves during the enzyme digestion was to change the composition of the enzyme solution. Instead of mannitol and high concentration of calsium chloride the use of sucrose and low concentration of calsium chloride allowed the leaves to digest without browning. Mannitol inhibited the protoplast-derived callus growth while sucrose and glucose allowed rapid growth. Similar effect of mannitol was also observed in fewerfew (*Tanacetum parthenium*) tissue culture where it decreased shoot growth and parthenolide production (Brown *et al*. 1996). Omission of mannitol from the medium also increased protoplast yield of *Helianthus annuus* (Moyne et al. 1988) and decreased the senescence of *Lactuca sativa* protoplasts (Oh et al. 1994). However, our result is in contrast to the previous study with *Chrysanthemum cinerariifolium*, where satisfactory leaf tissue digestion was achieved with an enzyme solution containing mannitol (Malaure et al. 1989). Calsium is also a factor in reducing enzymatic digestion in tomato (Bellini et al. 1990) and potato (Chang and Loescher 1991) tissues. In our study mannitol and calsium chloride were not studied separately, and thus we can only conclude that an enzyme solution supplemented with mannitol and calsium chloride may cause browning of tansy and pyrethrum leaf tissues during enzyme digestion, expecially if the source plants have been cultured under high light intensity. The deleterious effect of mannitol and high concentration of calsium ions for tansy and pyrethrum demonstrate the differences between species in their tissue culture behavior, because this solution has been successfully used for protoplast isolation from *Brassica* spp. (Christey et al. 1991).

Bacterial contamination

Occasional contamination became visible around roots of *in vitro* plantlets several months after the initiation of the cultures in our preliminary study (IV), a problem also observed in *Tanacetum parthenium* (Banthorpe and Brown 1989), led us to study this problem in more detail. Bacteria were isolated from the vicinity of the roots of nine *in vitro* grown tansy genotypes. All the bacteria were Gram-negative which is consistent with previous studies showing that the majority of bacterial contaminants in *in vitro* plantlets are Gram-negative (Brunner et al. 1995; Buckley et al. 1995; Peñalver et al. 1994). The lack of Gram-positive endophytic bacteria in tansy may be due to its volatile oils that according to other studies show bactericidal effect on Gram-positive bacteria but are less effective against Gram-negative bacteria (Holopainen and Kauppinen, 1989). Enterobacteriaceae such as *E. agglomerans*, and fluorescent pseudomonads such as *P. fluorescens* and *P. marginalis* (synonymous to *P. fluorescens*) are considered to be plant-associated because they have been isolated from many field-grown and/or micropropagated plants, e.g., *Delphinium*, *Gerbera*, *Hosta* and *Iris* (Leifert et al. 1989a), *Galega orientalis* Lam. (Valkonen et al. 1993), *Cynara scolymus* L. (Peñalver et al. 1994) and *Mentha* (Buckley et al. 1995). Several studies have shown that bacteria can colonize the vascular tissue (e.g. Valkonen et al. 1993; van Doorn et

al. 1991; Whitesides and Spotts 1991; Gardner et al. 1982) where they can be translocated (Whitesides and Spotts 1991).

Because growth medium used for plants may not be optimal for bacterial growth or may even inhibit the growth (Leifert et al. 1994), endophytically growing bacteria can remain undetected for a long time and over several subcultures of internodal cuttings or shoot tips. In this study, we observed that it could take up to five months before the contaminants could be detected in shoot cultures based on visible bacterial growth. Gentamicin, rifampicin and cefotaxime, and their combinations reduced the growth of Gram-negative bacteria isolated from the *in vitro* cultures of tansy. However, these antibiotics also had adverse effects on the growth of tansy shoot tips and callus cultures. Effects due to some antibiotics were more pronounced than those of others, and also the different tansy genotypes reacted differently. Gentamicin reduced the shoot and root growth more severely and caused more hyperhydricity (Debergh et al. 1992) than cefotaxime and rifampicin. In general, the effects of cefotaxime and rifampicin on plant growth were similar. However, at the antibiotic concentration of $\leq$180 mg l^{-1}, the slopes of the regression curves for initiation of shoots, number of shoots, growth rate, and the height of shoots were in all instances steeper with rifampicin than with cefotaxime. Therefore, at these concentrations, cefotaxime was less phytotoxic than rifampicin, and both were less phytotoxic than gentamicin. At concentrations of 180 - 230 mg l^{-1}, cefotaxime and rifampicin had similar effects, but at concentrations exceeding 230 mg l^{-1}, cefotaxime was more phytotoxic than rifampicin. The relative phytotoxities observed in our study were consistent with those previously reported on five other plant genera (Leifert et al. 1992).

The shoot number decreased by each of the antibiotics, which showed that antibiotics had a growth regulator-type effect. Previous studies on *in vitro* cultures of other plant species have shown that many antibiotics have hormone-like effects on plant growth (Leifert et al. 1992; Leifert et al. 1991; Santos and Salema 1989; Mukherji and Biswas 1979). For example, penicillin can stimulate the synthesis of cytokinins (Santos and Salema 1989), and ß-lactams increase the levels of phenylacetic acid (auxin) that is their break-down product (Holford and Newbury 1992). The auxin-type effect of rifampicin was clearly observed in our experiment with genotype *Tv* 5. Rooting increased as the concentration of rifampicin increased in the growth medium. Thus, the use of rifampicin in growth medium may overcome the effects of relatively high concentrations of the cytokinin, BAP that was used in our growth media and which usually prevent root formation.

In our subsequent tissue culture work with tansy we have added gentamicin, rifampicin and cefotaxime, or their combinations in the MS media for maintenance and propagation of tansy shoot cultures and found them effective in controlling bacterial growth. However, to avoid unwanted alterations in the growth of tansy the shoot cultures should be periodically (after 1-2 months) transferred to antibiotic free medium. The observed genotypic variation in the sensitivity of root growth to different antibiotics is also taken into account when we selected tansy genotypes for micropropagation and for protoplast cultures.

3 Protoplast technique for tansy and pyrethrum

Protoplast isolation and culture of protoplast derived-calli

Several factors affecting the protoplast isolation process were observed including seasonal effects on the viability and number of protoplasts isolated as well as various components in the protoplast culture medium and in the following proliferation conditions. There was a clear seasonal effect influencing the success of protoplast isolation and culture. The best period for protoplast isolation ranged from the end of December until the beginning of April. The underlying cause may be the quality or intensity of light. The growth chamber used had a transparent door through which natural light could passed in and supplement the fluorescent lamps. Similar effect of light intensity has been observed in protoplast cultures of *Brassica* (Zhao et al. 1995) and tomato (Bellini et al. 1990). The light effect was also observed in the pioneering protoplast studies on Petunia where the optimum season was the same as in our experiments (Frearson et al. 1973).

Protoplasts isolated from tansy showed sensitivity against a high concentration of ammonium ions and turned brown and died after one week. The inhibitory effect of ammonium ions on the growth of protoplasts has been previously reported in *Artemisia vulgaris* L., *Chrysanthemum indicum* and *C. zawadskii* (Okamura et al. 1984), *Chrysanthemum x hortorum* (Sauvadet et al. 1990), and *Helianthus annuus* (Lenee and Chupeau 1986).

Cells started to divide within 3 - 4 days at the optimum plating density of 0.2 - 0.4 x 10^6 protoplasts ml^{-1}. If the density of protoplasts was lower than 0.1 x 10^6 cells ml^{-1}, the protoplasts did not divide. High plating density was also observed to increase the division of *Senecio x hybridus* (Pillai et al. 1990) and *Cynara scolymus* L. (Ordas et al. 1991) protoplasts. The high NAA concentration of the protoplast culture medium used in the present research may have had a favourable influence on the division of tansy protoplasts as has been observed for other related species such as *Helianthus maximiliani* (Polgàr and Krasnyanski 1992) and *Helianthus annuus* (Krasnyanski and Menczel 1993). The protoplasts of *Cyanara scolymus* L. were also induced to divide by a high NAA and 2,4 D concentration (Ordas et al. 1991). Decrease of protoplast viability in the first few days after isolation has been a problem for protoplast culture of tansy (IV). In the present study red pigmentation in the protoplast culture was inhibited by the decrease in pH. Similarly, the decrease in pH during the first few days of protoplast culture inhibited cell division in *Helianthus annuus* (Lenee and Chupeau 1986) and *Lactuca sativa* (Brown et al. 1987) which could be overcome either by diluting the protoplast culture (Lenee and Chupeau 1986) or by adding buffer to the culture medium (Brown et al. 1997).

Different antibiotics were tested to decrease the loss of protoplast viability during the first and second week of culture as well as to stimulate regeneration of the protoplast derived callus. Low concentration of antibiotics delayed the decrease of protoplast viability which was probably due to the inhibition of the growth of endophytic micro-organisms which can be transferred from the field grown plants through micropropagated shoot tips to protoplast culture (III). The beneficial effect of antibiotics to protoplast division, viability and regeneration have also been observed by other authors for several plant species (d'Utra Vaz et al. 1993; Gilbert et al. 1991; Pollock et al. 1983). Interestingly, the growth rate of protoplast derived calli of tansy was increased when both gentamicin (25 mg l^{-1}) and

rifampicin (25 mg l^{-1}) were added to the growth media, whereas the use of either antibiotic alone reduced or did not have an effect on callus growth. In spite of the effect on callus proliferation, antibiotics did not stimulate shoot regeneration. Previous studies have shown that gentamicin can enhance callus growth and regeneration in *Catharanthus roseus* (L.) G. Don (Müllers and Sarkar 1989) whereas it is toxic to suspension cultures of *Datura innoxia* Mill. (Horsch and King 1983).

Experiments with different plant hormones showed that callus growth derived from tansy protoplasts and formation of globular structures did not occur without the addition of either auxin or cytokinin. Abundant callus growth was obtained with several auxin and cytokinin combinations and concentrations except when IAA and kinetin were added together. The largest calli were obtained from media including a combination of NAA & zeatin. The combination NAA & BAP enhanced the formation of globular structures on the calli which we expect to be meristematic and able to produce shoots. Pyrethrum protoplasts formed microcalli but their growth did not proceed further. Also a previous attempt to grow pyrethrum protoplast-derived calli ended at the callus stage (Malaure et al. 1989). Genotypes may differ in their regeneration ability which should be taken into consideration in future experiments. In this study we were able to use only one line of pyrethrum. However, the observed meristematic structures, root formation and sporadic shoot growth show that protoplast-derived calli of tansy have the potential to regenerate, although the development of a full protoplast-to-plant system still needs further studies. One reason for the limited regeneration may be incomplete cell partitioning which inhibits the signaling of hormones from cell to cell thus inhibiting differentiation (von Keller et al. 1994).

Protoplast fusion and analyses for the hybridity

Microcolonies and calli derived from protoplast fusion between tansy and pyrethrum grew more vigorously than calli derived from the intraspecific tansy fusion. Increased cell growth in interspecific hybrid callus may be due to heterosis which was also observed in the hybrids of *Lactuca sativa* x *L. virosa* (Matsumoto 1991) and in *Solanum brevidens* x *S. tuberosum* (Polgár et al. 1993). In the latter case the vigorous growth was used successfully as a selection marker for hybridity already at the callus level. The intraspecific fusion between two tansy genotypes produced very slowly dividing cells lacking green pigmentation in contrast with green and rapid dividing cells of tansy protoplasts (IV). Slowly dividing cells have been observed in protoplast fusion-derived *Senecio* hybrids which had chlorophyll-deficient spots in the stems associated with loss of chromosomes or fragments of chromosomes (Wang and Binding 1993). Similarly, abnormal chromosome number was associated with slow growth of intraspecific *Lactuca* hybrids (Matsumoto 1991).

Protoplast hybridity was tested by the analyses of nuclear DNA content, RAPD-PCR patterns and the composition of volatile compounds. The nuclear DNA content for tansy line *Tv* 14 was 7.84 pg (I), whereas for its sibling, *Tv* 142094, the nuclear content was 6.41 pg (VI) One reason for the difference in the nuclear DNA content may be that *Tv* 142094 is derived from a prematured seed whereas the *Tv* 14 used previously was micropropagated from a shoot tip excised from the field grown genotype *Tv* 14. Both tissue type and environmental conditions have been shown to influence nuclear DNA

content especially that of the repetitive sequences (Arnholdt-Schmitt 1993). DNA content of diploid nuclei of maize (*Zea mays* L.) varied between different plant tissues (Biradar and Rayburn 1993) whereas the 4C DNA content of sunflower (*Helianthus annuus* L.) was observed to vary depending on the site of the seed in the flower from which the regenerants were derived (Natali et al. 1998). Comparable 2C contents were detected from pyrethrum and protoplast-derived calli which were analyzed using the same procedure with two different flow cytometers. Interestingly, the chromosome complement 2n=2C=18 is the same for tansy (Virrankoski and Sorsa 1968) and pyrethrum (Pal 1992), but the nuclear DNA content of pyrethrum is about twice that of tansy. The nuclear DNA content of the calli derived from the two different types of fusions (tansy + tansy; tansy + pyrethrum) varied, but was more than the parental level in many fusion calli. The increased DNA content suggests that some degree of protoplast hybridization had occurred.

The genetic distance between the two tansy genotypes including the fusion program (0.381) was comparable to the range of the 20 tansy genotypes (0.294; I). The highest DNA content (19.59 pg, range 19.058 - 20.122) of callus F43C (tansy + tansy) indicates a fusion between three protoplasts. Genetic distance and cluster analysis suggest that the fusion may have occurred between two protoplasts of *Tv* 50 and one of *Tv* 142094. We arrived at this conclusion because the genetic distance from *Tv* 50 to the fusion callus (F43A) was closer than that from *Tv* 142094, and the clustering positioned the fusion callus (F43A) closer to *Tv* 50 that to *Tv* 142094. The nuclear DNA content (31.87 pg; range 30.315 - 33.425) measured for callus F46B (tansy + pyrethrum) suggest a fusion between more than two protoplasts or a double fusion. Because the average linkage method clustered the F46B callus closer to pyrethrum than to tansy, the fusion probably involved more of *Tc* 22 protoplasts. Also the DNA content of F46B is close to the sum of 2 x pyrethrum + 1 x tansy. In conclusion, several observations support the fact that fusions between tansy and pyrethrum occurred. Firstly, the genetic distances from tansy and pyrethrum to the fusion calli (F46B, C) are smaller than the distance between tansy and pyrethrum. Secondly, the genetic distances from both of the donor species to the calli are almost the same. Thirdly, the artificially mixed sample of tansy and pyrethrum DNA is more similar to the fusion calli than to either tansy or pyrethrum. Difficulties to regenerate the fusion calli in spite of several attempts may be a consequence of abnormal chromosome combinations, loss of chromosomes or chromosome fragments as observed also in other somatic hybrids (Wang and Binding 1993) explaining partly also the variation in the nuclear DNA contents.

Volatile compounds were analyzed from the parental tissues and fusion-derived calli. Most of the volatile compounds identified from tissue cultured tansy (*Tv* 142094) were similar to those detected from intact tansy flower heads (*Tv* 14) in another study of this thesis (II). The most significant difference was the absence of the major compound, artemisia ketone, from tissue cultured tansy (*Tv* 142094). Although, volatile compounds from leaves and flower heads have been comparable to each other (Hendriks et al. 1990; Holopainen et al. 1983), the different growth stage from which the volatiles were extracted (young tissue cultured shoots from *Tv* 142094, flower heads from *Tv* 14) may have had an affect. Another explanation for the divergent volatile composition regarding the major compound is, that *Tv* 142094 is derived from embryo from a flower of *Tv* 14 grown in the orchard. Due to high percent of cross-pollination (Lokki et al. 1973), combination with alien germplasm may have caused differentiated composition of volatiles in the sibling. ß-

Farnesene detected in pyrethrum in this thesis was recently found also from pyrethrum in another study (Maciver 1995). Interestingly, a sesquiterpene, germagrene D was identified from pyrethrum for the first time in this study, whereas several other germacranolides have been detected from pyrethrum by other authors (Maciver 1995; Sashida et al. 1983; Doskotch et al. 1971; Head 1969).

Volatile compounds such as syringaldehyde, coniferyl alcohol, and artedouglasia oxide were only detected from protoplast fusion-derived calli, the latter compound was also identified from intact tansy flower heads in another study in this thesis (I). Also other authors have observed that leaf calli of tansy do not contain compounds present in the parental plant but significant concentrations of novel compounds rare or not detected in an intact plant (Banthorpe and Brown 1989). In contrast, small content of thujone was observed from leaf derived callus of tansy (Svoboda et al. 1995). Growth conditions such as the type of carbon source and concentration (Brown et al. 1996; Rajasekaran et al. 1991; Banthorpe and Brown 1989), and hormones (Dhar and Pal 1993; Banthorpe and Brown 1990) have been reported to regulate the accumulation of terpenes in tissue culture derived calli from *Tanacetum* sp. The small number of volatiles detected from protoplast-derived calli of tansy and protoplast fusion-derived calli in this study indicated that the dedifferentiated callus seldom produces or accumulates terpenes (Brown et al. 1996; Banthorpe and Brown 1989; Banthorpe et al. 1986). Detection of a precursor compound for pyrethrins, chrysanthemic acid (Zito and Tio 1990; Kueh et al. 1985), was also an indication of the effect of dedifferentiated plant tissues on secondary metabolism. Similarly, the low number of compounds found from fusion-derived calli which are present in both tansy and pyrethrum may not indicate absence of hybridization, but rather the inability of non-organized tissue to produce volatile compounds.

VI CONCLUSIONS

Significant natural resources are contained in wild and under exploited species. To screen secondary metabolites from every species would not be meaningful, but combination of ideas from ethnobotany or traditional uses of plant species with modern technology can produce economical benefits in addition to the scientific interest and discoveries. Value creation from wild species is of interest especially for plant production in many industrialized countries where the demand for food can be met by yet a smaller group of farmers. High cost of production and problems associated with that are typical for countries such as Finland located close to the Arctic Circle where the growing season is very short and the crop yields rather low. As a consequence, either a significant part of the farming community will lose the economical opportunity to continue their work or alternative production methods are needed. Plant derived 'green chemicals', 'green bioenergy', and 'green fibres' are just examples of the germinating but increasing second 'green revolution' of the new millennium. The demand of the green chemicals containing products such as environmentally benign insecticides and medicines is increasing apart from the available synthetic alternatives.

In this thesis tansy was demonstrated to contain significant variation of interesting green chemicals, and thus as a species adapted to the Northern Hemisphere can be a candidate for a novel crop species. In Finland prospecting towards a crop which could be able to utilize the short growing season, species originating from geographical regions close to ours are of special interest.

Protoplast fusion technique has been applied in plant breeding to improve quantitative traits in many agronomically important crops. Protoplast fusion is also a novel method for improving crop quality. The possible routes for manipulating for example the isoprenoid pathway through protoplast fusion could be somaclonal variation, polyploidization, cellular compartmentalization of biochemical pathways, alteration of tissue types synthesizing isoprenoids or alteration in the isoprenoid pathway itself. The advantage of using protoplast fusion for metabolic engineering is that there is no need to know the pathways or enzymes responsible for the specific compounds and thus it is a good method for utilizing wild or under-exploited species. In this thesis somatic hybridization between tansy, a wild but climatically adapted species, and pyrethrum, a related species accumulating novel compounds, was carried out. The abundant callus growth and spontaneus root and shoot formation of protoplast-derived calli demonstrated the capacity of tansy to regenerate. Metabolic alterations of the somatic hybrid could not be observed at the callus level which can be a consequence of the non-differentiation of the tissue rather that the inability of the hybrid to produce novel compounds. Because variation, especially in the growth of different genotypes *in vitro* exists, the best regenerated genotypes of tansy and pyrethrum should be selected for future experiments. Cell partitioning may be a prerequisite for proper regeneration as observed in other Asteracea species and should be further studied in tansy. Tissues of tansy with high capacity to accumulate irregular monoterpenes such as artemisia ketone or davanone with high pyrethrin-producing lines of pyrethrum could also be of interest for future studies. In addition to volatile compounds, also less volatile sesquiterpenes and flavonoids imparting obviously also bioactivity in tansy could be studied not only for insecticidal activity but also for medicinal properties.

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