



Glandular trichomes from the leaves of *Artemisia tridentata vaseyana* (Rydb.) Beetle : ontogeny, ultrastructure, and terpenoid production
by Francoise Djibode

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University
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Abstract:

Terpenoids are produced by glandular trichomes of leaves of the mountain big sagebrush, *Artemisia tridentata vaseyana* (Rydb.) Beetle. Sagebrush possesses two types of leaves, summer ephemeral and winter persistent. I hypothesized that the peltate glandular trichomes of the two types of leaves were different and have a different pattern of producing terpenoids. Scanning electron microscopy and transmission electron microscopy were used to show the different types of trichomes of sagebrush leaves. Gas chromatography and mass spectroscopy were used for qualitative and quantitative analysis of the terpenoids in ephemeral leaves. Attempts to extract a fragment of the cyclase gene for study of the differential expression of cyclase mRNA in clipped versus control ephemeral leaves did not give satisfactory results.

Sagebrush has three types of trichomes: (1) "T"-shaped cover trichomes which blanket the leaves, giving them a silvery look. (2) Ten-cell peltate glandular trichomes on ephemeral leaves which are oblong in shape, and consist of one pair of stalk cells, one pair of secreting-support cells, and three pairs of secreting cells. This gland produces highly pressurized volatile terpenoids in the secretory head of the mature trichome and has four times the surface area as the round shaped trichome found on the persistent leaf. (3) Round trichomes on persistent leaves which have six pairs of cells consisting of one pair of stalk cells, one pair of support cells, and four pairs of secretory cells. These glands secrete volatile and non-volatile terpenoid compounds and are susceptible to shedding in extreme environmental conditions.

In three months, trichomes of the summer leaves reach their optimum production of volatiles, which makes this leaf type suitable for studies of alternatives for methyl bromide. The major terpenoid constituents of ephemeral leaves are camphor, eucarvone, eucalyptol (1, 8-cineole), and camphene. Camphor and eucarvone are natural pesticides, herbicides and fungicides. The increase of camphor (70%), eucarvone (66%), and α - and β -pinene 15 days after pruning, shows that *A. t. vaseyana* reacts to pruning of its branches. A mixture of camphor, eucarvone, eucalyptol, and α -pinene may be effective in controlling insect pests.

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VASEYANA (RYDB.) BEETLE: ONTOGENY, ULTRASTRUCTURE,
AND TERPENOID PRODUCTION

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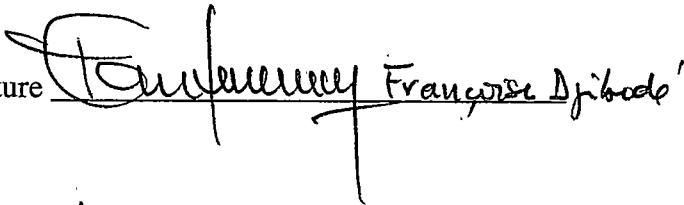
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ABSTRACT

Terpenoids are produced by glandular trichomes of leaves of the mountain big sagebrush, *Artemisia tridentata vaseyana* (Rydb.) Beetle. Sagebrush possesses two types of leaves, summer ephemeral and winter persistent. I hypothesized that the peltate glandular trichomes of the two types of leaves were different and have a different pattern of producing terpenoids. Scanning electron microscopy and transmission electron microscopy were used to show the different types of trichomes of sagebrush leaves. Gas chromatography and mass spectroscopy were used for qualitative and quantitative analysis of the terpenoids in ephemeral leaves. Attempts to extract a fragment of the cyclase gene for study of the differential expression of cyclase mRNA in clipped versus control ephemeral leaves did not give satisfactory results.

Sagebrush has three types of trichomes: (1) "T"-shaped cover trichomes which blanket the leaves, giving them a silvery look. (2) Ten-cell peltate glandular trichomes on ephemeral leaves which are oblong in shape, and consist of one pair of stalk cells, one pair of secreting-support cells, and three pairs of secreting cells. This gland produces highly pressurized volatile terpenoids in the secretory head of the mature trichome and has four times the surface area as the round shaped trichome found on the persistent leaf. (3) Round trichomes on persistent leaves which have six pairs of cells consisting of one pair of stalk cells, one pair of support cells, and four pairs of secretory cells. These glands secrete volatile and non-volatile terpenoid compounds and are susceptible to shedding in extreme environmental conditions.

In three months, trichomes of the summer leaves reach their optimum production of volatiles, which makes this leaf type suitable for studies of alternatives for methyl bromide. The major terpenoid constituents of ephemeral leaves are camphor, eucarvone, eucalyptol (1, 8-cineole), and camphene. Camphor and eucarvone are natural pesticides, herbicides and fungicides. The increase of camphor (70%), eucarvone (66%), and α - and β -pinene 15 days after pruning, shows that *A. t. vaseyana* reacts to pruning of its branches. A mixture of camphor, eucarvone, eucalyptol, and α -pinene may be effective in controlling insect pests.

CHAPTER 1

INTRODUCTION

Artemisia tridentata McArthur encompasses five subspecies including mountain big sagebrush *Artemisia tridentata* ssp. *vaseyana* (Rydb.) Beetle (Beetle, 1960; McArthur, 1979; Goodrich *et al.*, 1985). *Artemisia tridentata tridentata* Nutt., *A. tridentata vaseyana*, *A. tridentata wyomingensis* Beetle and Young, *A. tridentata lahotan* (Brunner) Winward, and *A. tridentata spiciformis* (Osterhout) are all members of the subspecies (Beetle, 1960; McArthur, 1979). The subgenus is natural with *A. tridentata* at its core but its limits are still under investigation because classical taxonomic methods do not reveal the evolutionary relationships among subspecies within the *Tridentatae* (Wilt *et al.*, 1992). *Artemisia t. vaseyana* was chosen for this study because of its low level of 2-methyl-2-propenal content compared to *A. t. tridentata*. Two-methyl-2-propenal is an extremely volatile irritant and bitter terpenoid which plays an important role in sagebrush choice by deterring browsing by wild mammals (Scholls *et al.*, 1977; Bray *et al.*, 1991). *Artemisia t. vaseyana* is the preferred taxon browsed during winter by mule deer because of its low content of 2-methyl-2-propenal (Personius *et al.*, 1987; Wambolt *et al.*, 1991; Weber *et al.*, 1994; Wambolt, 1996). All subspecies of *A. tridentata* exhibit two distinct types of stem growth: vegetative-perennial and reproductive-annual (DePuit and Caldwell, 1973). Two types of leaves are formed on the vegetative shoots during the course of the year. The primary new leaves which develop along the main branches at the beginning of spring

are large and typically sharply trilobed. As growth continues, new short lateral branchlets grow in August from the upper axil of the existing primary leaves. The branchlets support large quantities of smaller, less distinctly trilobed leaves which persist through the next winter, long after the large initial leaves are shed (Diettert, 1938; Goodwin, 1956; DePuit and Caldwell, 1973). The large primary leaves are shed from the end of June to September, depending on the environmental conditions and the developmental stage of the plant. The smaller leaves persist until the next spring when leaf growth for the new season commences, but they never persist for two entire growing seasons. The primary leaves are commonly called "ephemeral leaves", while the smaller leaves are termed "persistent leaves".

In the genus *Artemisia*, glandular trichomes are the secretion sites of the plant's natural products including terpenoids, coumarins and flavonoids (Buttkus *et al.*, 1977; Kelsey and Shafizadeh, 1980; Ascensão and Pais, 1982; Kelsey *et al.*, 1982; Duke and Paul, 1993). These substances have long been of interest to phytochemists because of their importance as flavoring, perfumery and pharmaceutical agents; they are also used for their pesticidal and herbicidal properties (Duke and Paul, 1993; Weaver *et al.*, 1995).

A number of glandular trichome secretions are thought to have a role in plant defense because of their toxicity, their deterrence to herbivorous insects, and their anti-fungal and anti-bacterial activity (Stipanovic, 1983; Kelsey *et al.*, 1984; Duke *et al.*, 1988). Contents of sagebrush trichomes have been extracted, identified and quantified (Kelsey *et al.*, 1982; Cedarleaf *et al.*, 1983). According to Cedarleaf *et al.* (1983), monoterpenoid content of the leaves is the highest in July (4.18% of dried leaves) when

only the large leaves are present; monoterpenoids thereafter decline in winter and reach the lowest level (0.97% of dried leaf in weight) in May of the following year.

Monoterpenes (Welch and McArthur, 1981) and sesquiterpene lactones (Kelsey and Shafizadeh, 1979, 1980) are the major secondary metabolic products in sagebrush, with lower quantities of coumarins (Shafizadeh and Melnikoff, 1970; Brown *et al.*, 1975) and flavonoids (Rodriguez *et al.*, 1972). Most of the studies have been focused on the terpenoid content of the persistent leaves, because the sagebrush leaves are used by wild animals as food during winter time when only persistent leaves are available (Longhurst *et al.*, 1969; Scholl *et al.*, 1977; Welch and McArthur, 1986; Bray *et al.*, 1991). Several studies have shown that high levels or composition of various proportions of terpenoids reduced rumen microbial activity of browsing species (elk, mule deer, and pronghorn antelope) (Personius *et al.*, 1987; Bray *et al.*, 1991).

Monoterpenoids have been demonstrated to act as toxins and feeding deterrents to a large variety of insects. They thus appear to have important roles in protecting plants from insect attack (Zalkow *et al.*, 1979; Saxena and Basit, 1982). However, some studies describe cases in which herbivorous mammals or insects appear to be immune to the noxious effects of terpenoids; and these substances then serve as attractants, feeding stimulants, or oviposition cues in these organisms (Radwan and Crouch, 1978; Hanson *et al.*, 1986).

Chemical changes induced by herbivory could be active responses to discourage further herbivory (Green and Ryan, 1972; Carroll and Hoffman, 1980; Edwards and Wratten, 1982; Schultz and Baldwin, 1982; Fowler and Lawton, 1985). Preliminary

evidence suggested that these chemical changes inhibited herbivore growth and development, and decreased herbivore population growth, thus reducing herbivore pressure on the plant. There are fewer reports of induced defenses among terpenoids than among other classes of secondary metabolites, especially in leaf tissue. This may be due to the existence of physiological constraints on terpenoid biosynthesis in mature leaves. Terpenoid biosynthesis is generally restricted to trichomes that are active early in leaf development (Fahn, 1979). Thus, evidence for the induced responses of terpenoids to herbivory should be sought not only in leaves present at the time of damage but also in leaves initiated immediately after damage has occurred (Coleman and Jones, 1991; Tallamy and Raupp, 1991). Another study suggested that sagebrush responded to any leaf herbivory by leaf-chewing arthropods by altering the chemical composition of leaves (Wiens *et al.*, 1991).

Sagebrush Origin, Systematics and Evolution

Artemisia L. is one of the genera of the Tribe (ensemble of genera) Anthemideae (Family Asteraceae) which consists of over 400 species including wormwood, sagebrushes, mugworts, sageworts, worseed and tarragon (Beetle, 1960; McArthur, 1979; Duke *et al.*, 1988). *Artemisia* is the most specialized and advanced genus in the Anthemideae. A number of characteristics are indicative of the advanced evolutionary position of *Artemisia* in that tribe. The inflorescences are reduced to small capitula consisting of two to twenty, mostly discoid and perfect florets. The arrangement of the capitula in elongate, narrow panicles characterizes *Artemisia* relative to other genera in the

Anthemideae. The taxa are all wind-pollinated and the elongation of the inflorescence is an effective means of exposing pollen and stigma to wind movement (Carlquist, 1966). Wind pollination (Thorne, 1973) in members of this genus is good evidence that the woody members are a specialization compared to taxa from the herbaceous ancestors in the same genus which are devoid of woodiness (Carlquist, 1962).

Progenitors of *Artemisia* probably evolved in the mesic, temperate habitats of mid-eastern Asia during the late Miocene to early Pliocene. *Artemisia* of the Eurasian subgenus *Sephiridium* (Bess.) Rouy occurs in the area immediately east of the Caspian Sea, where there are as many as 35 species (Person, 1974). Krasheninnikov (1946) had defined a Chino-nipponese region as the area with the highest species diversity, including the herbaceous members, out of the six principal centers of Eurasian *Artemisia*. The North-American members of *Artemisia*, including the sagebrushes, are likely derivatives of the subgenus *Dracunculus* (Bess.) plants that bridged Beringia from Asia to North America. Early systematic treatment combined all woody North American species of *Artemisia* with the Old World woody members of the subgenus *Sephiridium*. Recent chemical (Kelsey and Shafizadeh, 1979), cytological and chromosomal karyotype studies (Moss, 1940; Giessman and Irwin, 1974; McArthur *et al.*, 1981) support the segregation of North American taxa as the subgenus *Tridentatae*.

Artemisia subgenus *Tridentatae* is the most successful group of shrubs within the cold desert region of western North America. This group covers an estimated 68 million hectares, primarily in the region known as the Great Basin. *Artemisia t. vaseyana* extends from 31° N parallel in Baja California, north into British Columbia, south and east through

the Great Basin and the Rocky Mountains, and into the southwestern states of Arizona and New Mexico (Beetle, 1960). It is primarily encountered below 1750 m elevation, and in a zone of precipitation from 150 mm to 500 mm per year (Beetle, 1960).

The subgenus *Tridentatae* is composed of 11 species of differing geographic distribution (McArthur and Plummer, 1978) and taxonomic importance (McArthur, 1979). They are: *Artemisia arbuscula* Nutt., *A. argillosa* Beetle, *A. bigelovii* Gray, *A. cana* Pursh, *A. rothrockii* Gray, *A. tridentata* Nutt., *A. tripartita* Rydb., *A. pygmaea* Gray and *A. rigida* (Nutt.) Gray (Beetle, 1960; McArthur, 1979). Four are further divided into subspecies: *A. arbuscula* (2 subspecies), *A. cana* (3 subspecies), *A. tridentata* (5 subspecies), *A. tripartita* (2 subspecies). Few species of the genus *Artemisia* and no other *Tridentatae* occur in South America (McArthur, 1979).

Development and Longevity of the Sagebrush Leaves

Shrub success in its range may be attributed in large part to the dimorphic development of its leaves. DePuit and Caldwell (1973) suggested that the ability of the plant to maintain part of the leaves through the winter enables it to begin growth and utilize water in the early spring. Development and maintenance of large ephemeral leaves during optimal growing conditions may also increase photosynthetic potential by reducing mesophyll resistance (DePuit and Caldwell, 1973). Confusion exists regarding the longevity of ephemeral and persistent leaves. On the basin big sagebrush, *A. t. tridentata*, growing east of the Rocky Mountains, mature leaves remaining on the plant over winter are discarded soon after spring growth resumes (Diettert, 1938; Branson *et al.*, 1976). On

three subspecies of big sagebrush growing in the Great Basin (*A. t. tridentata*, *A. t. vaseyana*, and *A. t. wyomingensis*), persistent leaves remain green on the plant throughout the subsequent growing season. These leaves senesce during initiation of summer drought, concurrent with abscission of the large ephemeral leaves (Miller *et al.*, 1986). Miller and Schultz (1987) studied the ontogeny, development, and longevity of both kinds of leaves from *A. t. wyomingensis* for two years. The study concluded that the big sagebrush is a semi-evergreen shrub, which maintains a portion of its leaves through the winter. Longevity of the persistent leaves is in the range of 12 to 13 months, with no leaves persisting through two winters (Miller and Schultz, 1987). Winter-persistent leaves did not reinitiate elongation the subsequent spring. The large ephemeral leaves are the first to develop early in the spring from small leaf buds at the apex of the branches. Ephemeral leaf elongation begins in early spring, forming tight clusters or fascicles at stem apices prior to stem elongation. When branches begin to elongate, ephemeral leaves are alternately positioned along the branch. These early ephemeral leaves are the largest leaves on the plant (Diettert, 1938; Miller and Schultz, 1987). As spring progresses and the early ephemeral leaves are near maturity, a small cluster of leaves begins to develop in the axil of each ephemeral leaf. These fascicles contain both ephemeral and persistent leaves. Lateral leaf fascicles are properly termed "short shoots". Each short shoot fascicle is subtended by a long shoot and one large lateral leaf (Miller and Schultz, 1987). Later developing ephemeral leaves are smaller than the early ephemeral leaves, but larger than the fully expanded persistent leaves (Miller and Schultz, 1987). Ephemeral leaves on the reproductive stem are not lobed and have no short shoot fascicle in their axes. At the

onset of drought, both the persistent leaves of the previous season and the large early ephemeral leaves of the current year begin to senesce. Once the water potential in the wettest soil drops below -0.2 MPA, ephemeral leaves begin to senesce and long shoot elongation stops. This generally occurs at the end of July through August (Miller and Schultz, 1987). This pattern of growth is encountered in all taxa belonging to the *Tridentatae* subspecies.

No reports have been published showing the difference between the glandular trichomes of the large leaves and the small leaves, which appear to be physiologically and anatomically different, and might explain reduced terpenoid content of small leaves that serve as animal forage during the winter. There are no published reports showing terpenoid content in sagebrush ephemeral leaves after clipping of the branches. Likewise there are no reported investigations of the genetic control of the induced response of the monoterpenoid content of the leaves of a greenhouse-grown *A. t. vaseyana* plant.

Objectives of the Study

The objective of this study is to investigate the possible use of volatile terpenoids from the mountain big sagebrush (*A. t. vaseyana*) as alternatives to methyl bromide in agriculture. Methyl bromide is an effective herbicide, nematicide, insecticide and fungicide that has been used commercially for soil fumigation and quarantine (post-harvest) purposes for most of the twentieth century (Ragsdale and Wheeler, 1995). Considerable evidence has been accumulated that methyl bromide is a potent compound that contributes to the depletion of the ozone layer, and is scheduled to be phased out in the U.S.A. by

2005 under the Clean Air Act (United States Environmental Protection Agency, 1993). Over 90% of the earth's ozone is present in the stratosphere that extends from 16 to 160 km above the earth's surface. Stratospheric ozone provides a protective layer for the earth's surface by absorbing ultraviolet radiation from the sun, thus moderating the climate on our planet (World Meteorological Organization, 1994). Atmospheric pollutants such as chlorine (photolysis product of chlorofluorocarbons) and bromine break down stratospheric ozone molecules by reacting with them chemically (Molina and Rowland, 1974). At least seven organic bromine compounds have been identified in the atmosphere (Penkett *et al.*, 1985). Bromoform emitted by ocean sources is the largest contributor to atmospheric bromine, but has a short life due to photolysis (Penkett *et al.*, 1985). Methyl bromide is the major carrier of bromine to the stratosphere (Penkett *et al.*, 1985). It breaks down to form bromine which not only reacts with the ozone molecule, but it is also 50 times more reactive than chlorine in depleting ozone by combining with reservoir chlorine species, freeing the chlorine to react with additional ozone (World Meteorological Organization, 1994; United Nations Environment Programme, 1995; Cox *et al.*, 1995). It has become important to discontinue methyl bromide production and find alternative compounds for use in agriculture.

Hypotheses

I hypothesized that:

- 1) The glandular trichomes of the two types of leaves have different anatomy and physiology.

- 2) The ephemeral leaves respond to clipping and defoliation by increasing the quantity and quality of their terpenoid content.
- 3) Terpenoid production by the sagebrush is under genetic control, and is stimulated by clipping or defoliation.

In this dissertation I examine the ontogeny and ultrastructure of the glandular trichomes from both ephemeral and persistent leaves from greenhouse grown plants of *A. t. vaseyana*. The terpenoids produced by ephemeral and persistent leaves were analyzed. I also address the evolutionary significance of the plant in terms of herbivory by mammals and insects. The study of volatile compounds produced by the ephemeral leaves after clipping of the branches was also done for the ultimate goal of using analogs of the volatile compounds emitted by ephemeral leaves as an alternative to methyl bromide for the control of post-harvest and soil borne pests. An attempt was made to isolate a fragment of terpenoid synthase from the large leaves of the sagebrush plant. Such a gene fragment can be used to test the differential expression of terpene produced in response to clipping of branches.

CHAPTER 2

TRICHOMES OF THE LEAVES OF *ARTEMISIA TRIDENTATA VASEYANA*:
ONTOGENY AND ULTRASTRUCTUREIntroduction

Glandular trichomes which secrete lipid material have been described as occurring on leaves and flower parts of a large number of genera in the plant families Asteraceae, Lamiaceae, and Geraniaceae (Haberlandt, 1914; Metcalfe and Chalk, 1950; Cutter, 1969). The glandular trichomes of the genus *Artemisia* from the Asteraceae are the accumulation sites of a variety of terpenoids. The major composition of the terpenoids secreted in the leaves from the sagebrush plant are monoterpenoids and sesquiterpenoids. There is considerable interest in the exploitation of the terpenoids secreted by the sagebrush as herbicidal and anti-herbivore agents. Sagebrush is a perennial plant which possesses two types of leaves: the ephemeral leaves which are present only in summer during a period of increased terpenoid production, and the persistent leaves which are mostly present in winter and show a monthly decrease in the production of terpenoids (Cedarleaf *et al.*, 1983).

Previous Studies of Trichomes from the Genus *Artemisia*

The non-glandular trichomes of the genus *Artemisia* are uniseriate, with a multicellular nucleated stalk topped by an enucleate unicellular "arm" which is 400-500 μm long and present in both adaxial and abaxial surfaces of the leaf (Diettert, 1938).

There is an interspecific variation in non-glandular trichome density, from lower density in *A. nova* and *A. rothrockii* to no cover of trichomes in *A. pygmaea*, a taxon which compensates its lack of protection from the trichome layer by an increased cuticular thickness (Shultz, 1983). The two-armed, stalked trichomes were termed "cover trichomes" by Vesque and Viet (1981), due to the manner in which these trichomes blanket the leaf surface. The presence of these air-filled trichomes appears to be significant in an arid climate (Shultz, 1983). The transpirational rates were measured from pubescent and glabrous surfaces by Ehleringer *et al.* (1976). They concluded that a hairy coat is more effective in reducing transpiration in the presence of wind, and is also more effective than a heavy cuticle in reducing water loss in windy environments where ground water is abundant. The effect of leaf pubescence on photosynthesis was also investigated by Ehleringer *et al.* (1976) and Ehleringer and Mooney (1978); they concluded that pubescence modifies the heat load of the plant by increasing light reflectance, thus maintaining leaf temperature at a thermal optimum for photosynthesis.

Glandular trichomes from *A. tridentatae* have been studied using light microscopes (Diettert, 1938; Kelsey and Shafizadeh, 1980). The ten-celled peltate gland from the genus *Artemisia* is composed of one pair of stalk cells; and four pairs of cells filled with secretory material (Diettert, 1938). No distinction was made as to whether the leaves were ephemeral or persistent but if it was 10-celled, it must only have been from the ephemeral leaves. The biseriate peltate glandular trichome of *Artemisia nova* is filled with sesquiterpene lactone enclosed in a membranous sac (Kelsey and Shafizadeh, 1980). The gland from *A. nova* is typically 25 to 30 μm wide in transection. Differentiation of

epidermal cells into trichomes begins when the foliar primordium has reached a length of 80 μm (Diettert, 1938). Glandular trichomes are found throughout the plant, on young stems, leaves, inflorescence bracts, corollas, and achenes of *A. nova* (Slone and Kelsey, 1985).

Secretion of Terpenoids

Terpenoids are biosynthesized *in situ* by the glandular trichomes. Gland cells frequently display ultrastructural features (active chloroplasts or plastids) that suggest active metabolism of gland products (Fahn, 1979). Direct evidence for the biosynthetic capabilities of gland cells has become available. Studies with tobacco (*Nicotiana tabacum* L.), the glandular trichomes of which secrete principally divane-type diterpenes and sucrose esters, have demonstrated that gland cells incorporate basic metabolic precursors into both of these types of glandular substances (Keene and Wagner, 1985; Kandra and Wagner, 1988). Spearmint (*Mentha spicata* L.) plants accumulate an essential oil containing principally monoterpenes in their glandular trichomes. The enzymes of monoterpene biosynthesis were shown to be located only in the cells of the glandular trichomes and not in the remainder of the leaf (Gershenson *et al.*, 1989).

Cell cultures of *Artemisia annua* L. failed to produce artemisinin, a compound which has potent antimalarial activity (Duke *et al.*, 1994). The lack of artemisinin biosynthesis in cell culture is due to the fact that the sesquiterpene lactone artemisinin is produced in the glandular trichome, not in other parts of the plant (Duke *et al.*, 1994). Glandless leaves of *A. annua* could not produce artemisinin (Duke *et al.*, 1994). Thus,

different techniques have been developed to obtain highly-purified preparations of glandular trichomes, for the *in vivo* study of the different pathways of terpenoid synthesis in the whole intact glands (Slone and Kelsey, 1985; Gershenson *et al.*, 1987).

The objective of this part of the study was to examine the ontogeny and pattern of volatile production, to show the differences between the peltate glandular trichomes of the ephemeral and the persistent leaves of *A. t. vaseyana*, and to discuss the implication for pesticide research.

Materials and Methods

Plant Material

Seeds of *A. t. vaseyana* were collected in November, 1995, from the study site in the Gallatin Valley, at an altitude of 1550 m on a southwestern facing slope in T2S, R4SW, approximately 8 km northeast of Bozeman, Montana. The seeds from a plant tagged #3 showed the highest germination rate (90-97%) and were retained for further experiments. Seeds from plant #3 vernalized at 4 °C for 10 days in a wetted Whatman Paper No. 3 were sown on 24 January and 23 June, 1996, one per cell in a 72-cell seed bed (No. 1206 insert for No. 1020 flat 11" x 22" T. O. plastics, Belden Plastics, St. Paul, MN), filled with pre-wetted Aqua 2000 potting media (one part of Bozeman silt loam, one part of peat moss, one part of washed concrete sand, and 1lb per cubic yard of Aqua Gro 2000G). The ensemble was covered with a clear plastic dome. The plants were watered with tap water once every two days after seedling establishment. In addition, they were watered twice per week with a dilute solution of Peter's 20-10-20 general purpose

fertilizer (0.25 g/l). The seedlings were transplanted after two months into 60-cubic-inch pots (Stuewe & Sons, Inc., Corvallis, OR) where they were grown for one year. One-year-old plants were transplanted into 8-inch pots (AZALEA Pot, Belden Plastics, St. Paul, MN) for further growth. Leaves from mature plants (30 to 90 days for the ephemeral leaves; 30 to 365 days old for persistent leaves) were sampled and prepared for microscopy studies.

Leaf Size

Leaf Size Measurement. Leaves from 24 plants, grown from 24 January to 21 October, 1996, were harvested. One ephemeral leaf and one persistent leaf from each plant were harvested from the topmost section and the base of each harvested branch, respectively. A total of 48 ephemeral and 48 persistent leaves was measured with a ruler. Leaf length (including petiole), petiole length, and leaf width (at the widest point) were measured to compare the ephemeral and the persistent leaves.

Leaf Surface Area Measurement. Twenty-seven ephemeral and 29 persistent leaves were randomly selected from four plants. Their surface area was measured using Carl Zidas's (1985 Carl Zeiss Inc. Heidelberg, Germany) thin pad. The leaves were immobilized on the console of the thin pad using clear tape. The mean area per leaf type was computed.

The length and the width of the transmission electron microscope print of one mature glandular trichome from an ephemeral leaf and one print from the mature trichome

of a persistent leaf were measured three times with Carl Zidas's thin pad. The thin pad was set with the magnification of each print, and the surface area of each type of trichome was automatically shown on the paper printed out from the printer of the thin pad. These measurements of the two types of trichomes permitted comparison of the area of the two types of mature peltate trichomes.

Size of Peltate Glandular Trichomes

Determining Trichome Number on Ephemeral Leaves. Three-month-old ephemeral leaves harvested from many plants were cut into 1 mm squares and placed in a rigid plastic container (50 ml polypropylene centrifuge tube with screw cap), capped loosely, and frozen quickly in liquid nitrogen (-190°C). The tube was loosely capped to prevent shattering by the temperature differential and to allow some liquid nitrogen to flow into the tube and freeze the leaf tissues. The tube was removed from the liquid nitrogen tank and 3 cm³ of finely powdered dry ice (prepared by wrapping a piece in clean paper towels and crushing with a hammer) were added to the tube. The tube was loosely capped and vortexed using a model G-560 Vortex-Genie 2 mixer (Scientific Industries, Bohemia, NY) at a maximum speed of 3200 rpm for 1 min (Yerger *et al.*, 1991). The thick cover trichomes were sheared off the surface of the leaf tissues and the number of peltate glandular trichomes from the 27 samples were counted using a dissecting microscope (x 600). One sample was photographed to show the organization of the trichomes on the surface of the leaf (Figure 1).

