POLYACETYLENES FROM *BIDENS*

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ABSTRACT

The Hawaiian species of *Bidens* are morphologically and ecologically diverse taxa which have evolved from a single ancestral species. Adaptive radiation has occurred without the evolution of physiological or genetic interspecific isolating mechanisms since all species are interfertile and genetic distances among populations, based on isozyme loci, show little correlation with morphological differences or taxonomic classification. This disparity between the evolution of morphological and biochemical characters makes it of interest to determine whether or not there has been divergence in secondary metabolites in these species.

Leaves and roots of 19 species and six subspecies of Hawaiian *Bidens* were examined for polyacetylenes. Eleven C_{13} hydrocarbons, aromatic and thiophenyl derivatives, one C_{14} tetrahydropyran and three C_{17} hydrocarbons were isolated and identified. All can be derived from oleic acid. Polyacetylenes were not detected in the leaves of 13 taxa although they are found in the roots of all species. The occurrence of 2-(2-phenylethyne-1-yl)-5 acetoxymethyl thiophene in *Bidens* has not been previously reported. Most taxa could be distinguished by their complement of leaf and root acetylenes and no variation was found within taxa except in *B. torta*. There appears to be no taxonomically significant pattern to the distribution of polyacetylenes above the species level in this group.

The complexity of polyacetylene inheritance was assessed using experimentally produced interspecific hybrids. Crosses between species which do not produce leaf acetylenes resulted in F_1 individuals without acetylenes. Crosses between species which produce leaf acetylenes and those which do not yielded hybrids with acetylenes not always identical to parental arrays. Progeny from parents with different sets of acetylenes expressed a combination of the major compounds found in both parents. In all cases, nonparental acetylenes in the F_1 generation were biosynthetically closely related to compounds found in the parents. Polyacetylene synthesis was not segregated in the F_2 individuals from Type B crosses.

De novo biosynthesis of polyacetylenes in *Bidens* leaves was investigated in pulse-chase studies. ^{14}C-labelled acetylenes were recovered from three species of *Bidens* administered $^{14}CO_2$ and subsequently allowed to metabolize in $^{12}CO_2$ for 12, 24 and 168 hours. Radioactive C_{13} ene-tetrayne-ene was also isolated from the roots of all plants, indicating that translocation of ^{14}C-labelled precursors from aerial tissues occurred.

Phenylheptatriyne (PHT) was detected in two day old seedlings of *B. alba*, suggesting that polyacetylene biosynthesis begins during germination or soon thereafter. Quantities in the leaves continue to increase up to and beyond 24 days while amounts in the hypocotyls peak at seven days. Relative PHT values in the roots are 100 times higher than those in the aerial tissues for the first 24 days, but there is also a gradual decline in these levels beginning at two weeks and continuing beyond the experimental period. Phenylheptatriyne is absent from the roots of mature *B. alba*.

Many polyacetylenes are toxic to biological systems in the presence of UV-A radiation. These *in vitro* effects have led to speculation about the putative functions of polyacetylenes in the organisms which produce them. Nineteen species of phylloplane yeasts and yeast-like fungi were isolated from species of Hawaiian *Bidens* with and without leaf acetylenes. Although all these organisms, members of the Sporobolomycetaceae, Cryptococcaceae and Fungi Imperfecti, were photosensitive to some polyacetylenes and resistant to others, there was no correlation between the presence or absence of leaf polyacetylenes and the distribution of these saprophytes among species of *Bidens*. Nevertheless, it is significant that the only pathogenic species isolated in this study, *Colletotrichum gloeosporiodes*, did not colonize *Bidens* leaves containing C_{13} aromatic acetylenes to which it is extremely photosensitive *in vitro*.

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I. GENERAL INTRODUCTION

A. POLYACETYLENES

The majority of natural acetylenes known today are polyacetylenes. The name encompasses what now appears to be a biogenetically uniform group of secondary metabolites, usually not strictly poly-ynes (Jones and Thaller, 1978), which originate from oleic acid (Bu'Lock, 1966) and are found in the roots and aerial parts of plants, and in fungi (Bohlmann *et al*., 1973), algae (de Napoli *et al*., 1981), sponges (Cimino *et al*., 1981), nudibranchs (Walker and Faulkner, 1981), sea hares (Schulte *et al*., 1981) and insects (Moore and Brown, 1978).

Historically, the occurrence of a triple bond in a natural product was first clearly established by Arnaud (1902) in his study of the monoacetylenic acid, tariric acid (Table I), a component of the seed fat of *Picramnia tariri* DC. (Simaroubaceae). The first aromatic compound, carlina oxide (Table I), was isolated and studied by Semmler (1906), who, considering the natural occurrence of a triple bond unlikely, proposed an allenic formula for the compound. The correct structure was given by Gilman *et al*. (1933). The structural elucidation of a naturally-occurring *poly*acetylene was first achieved by Vil'yams *et al*. (1935) who recognized the lachnophyllum ester isolated from *Lachnophyllum gossypinum* Bge. as the methyl ester of dec-2-ene-4,6-diynoic acid (Table I). These first compounds were found accidentally because they were present in reasonable amounts and easily purified.

Seven acetylenes were described between 1902 and 1950. since then, over 700 more have been identified (Thaller, 1976) primarily because aliphatic polyacetylenes were discovered to show very characteristic UV spectra with high extinction coefficients, thus allowing detection of small quantities of substance (Jones, 1959; 1966; Bohlmann *et al*., 1973).

Around 1950, antibiotic substances produced by Basidiomycetes were characterized by UV spectra showing fine structure, which, by comparison with polyacetylenes from Compositae, tentatively established their acetylene structures (Anchel *et al*, 1950; Anchel, 1953; Kavanagh *et al*; 1950). Serious attention was focussed on this area when Celmer and Solomon (1952a; 1952b; 1953) isolated and identified the antibiotic mycomycin as one containing allene, diacetylene and diene groupings (Table I).

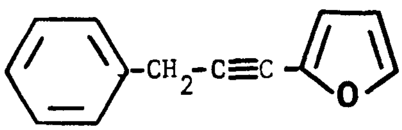
When Jones and his co-workers started their broad investigations into acetylenes from fungi, they changed the screening technique from an antibiotic test to one of determining the UV spectra of culture fluids (Jones, 1959). It is largely through the concurrent efforts of Jones, Sørensen, Bohlmann, Anchel and their associates in the last 30 years that so many polyacetylenes are known today. About 85% of these were isolated from higher plants. They are fairly widespread amongst the Campanulaceae and Araliaceae

TABLE I. NATURALLY OCCURING ACETYLENES

$$CH_3-(CH_2)_{10}-C\equiv C-(CH_2)_4COOH$$

tariric acid

Picramnia tariri DC.



carlina oxide

Carlina acaulis L.

$$CH_3-(CH_2)_2-(C\equiv C)_2-CH=CH-COOCH_3$$

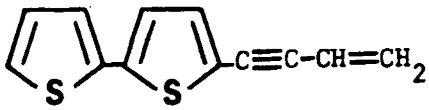
lachnophyllum ester

Lachnophyllum gossypinum Bge.

$$H(C\equiv C)_2-CH=C=CH-(CH=CH)_2CH_2COOH$$

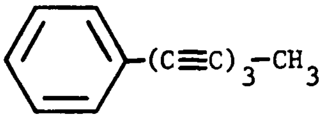
mycomycin

Nocardia acidophilus



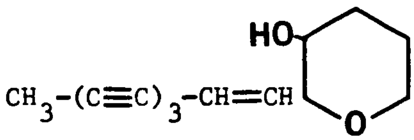
5-(3-buten-1-ynyl)-2,2'-bithienyl

Tagetes patula L.



phenylheptatriyne

Bidens alba L.



ichthyothereol

Ichthyothere terminalis Spreng.

$$HO{-}CH_2{-}(CH_2)_2{-}(C{\equiv}C)_2{-}(CH{=}CH)_3{-}CH\ (OH){-}(CH_2)_2{-}CH_3$$

cicutoxin

Cicuta virosa L.

$CH_3{-}CH{=}CH\ (C{\equiv}C)_4{-}CH{=}CH_2$

C_{13}-ene-tetrayne-ene

Heliantheae: Coreopsidinae

$CH_3{-}(C{\equiv}C)_5{-}CH{=}CH_2$

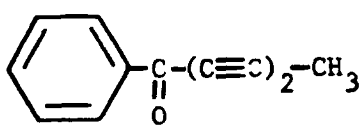
C_{13}-pentayne-ene

Heliantheae: Coreopsidinae

$CH_2{=}CH{-}CO{-}(C{\equiv}C)_2{-}CH_2{-}CH{=}CH{-}(CH_2)_5{-}CH{=}CH_2$

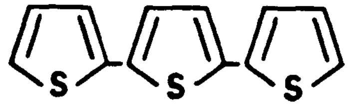
dehydrofalcarinone

Heliantheae: Galinsoginae



capillin

Artemesia capillaris Thnb.



α-terthienyl

Tagetes patula L.

and have been found sporadically in several other plant families (Bohlmann *et al*., 1973). They are most frequently found in members of the Umbellifereae and the Compositae, in which they occur in all 13 tribes, especially the Heliantheae, Anthemideae and Cynareae (Sørensen, 1977; Swain and Williams, 1977).

As distinct from the mainly aliphatic acetylenes of related plant families, acetylenes of the Compositae are characterized by cyclic, aromatic or heterocyclic end groups. Some of these complex structures are restricted to a single tribe while some heterocyclic compounds, such as thiophenes, have been found in the majority of tribes, their occurrence seemingly unrelated to morphological characters (Sørensen, 1977). In fact, the distribution of acetylenes in the Compositae does not often correlate well with botanical classification. Bohlmann *et al*. (1973) have made an extensive study of the polyacetylenes from this family and it appears that, although acetylene distribution is discrete, particular features are mostly restricted to some subtribes, some genera or some sections, so that polyacetylenes may be useful taxonomically at different levels below the tribe.

For example, members of the subtribe Heliantheae: Coreopsidinae are characterized by C_{13} ene-tetrayne-ene (Table I) and its aromatic derivatives, together with less unsaturated compounds. The subtribe H: Galinsoginae contains dehydrofalcarinone and other C_{17} acetylenes (Table I)

(Bohlmann *et al.*, 1973). Within Coreopsidinae, *Coreopsis*, *Bidens* and *Dahlia*, closely related genera, have similar acetylene arrays (Bohlmann and Zdero, 1968; Bohlmann and Bornowski, 1966; Bohlmann *et al.*, 1967; 1966; 1964; Sørensen and Sørensen, 1966; 1958a, 1958b, 1958c; Sørensen *et al.*, 1961), except that *Dahlia* also has C_{17} acetylenes (Lam 1971; 1973; Lam and Kaufmann, 1971; Chin *et al.*, 1970) which are characteristic of two other related genera *Glossocardia* and *Isostigma* (Bohlmann *et al.*, 1973).

The first fungal polyacetylenes, like mycomycin (Celmer and Solomon, 1953) and agrocybin (Bohlmann *et al.*, 1969), were detected and isolated because of their antibiotic properties. In the same way, the discovery of antibiotic properties of plants or of plant extracts has led to the identification of polyacetylenes as the active compounds. Thus the antifungal compounds from *Artemesia capillaris* Thunb. were identified as conjugated acetylenic ketones such as capillin (Table I) which is highly active against dermal mycoses (Jones and Thaller, 1978; Wagner, 1977).

Reisch *et al.* (1967) investigated the bacteriostatic and fungistatic effects of a large number of simple synthetic acetylenes, including hydrocarbons, acids, alcohols, aldehydes and ketones with one or two triple bonds, as well as the C_{13}-ene-tetrayne-ene and pentayne-ene compounds. In general their findings suggest that acetylenes with aromatic substituents were most active and that fungicidal effects increase with polarization of the triple bond and degree of unsaturation in the molecule while compounds which were more hydrophilic tend to be bacteriocidal agents.

Several polyacetylenes are known to be generally toxic. The plant extract used by natives of the Lower Amazon Basin as fish poison on their arrowheads contains the tetrahydropyran ichthyothereol and its acetate (Table I) as its active principles (Cascon *et al.*, 1965) and the potent toxicity of *Cicuta virosa* L. is due to cicutoxin (Anet *et al.*, 1953).

In 1973, Gommers and Geerligs reported that the nematocidal activities of α-terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl (Table I) were significantly enhanced by UV light. These compounds were subsequently isolated from *Tagetes patula* L. and found to be phototoxic to *Candida albicans* (Robin) Berkh. (Daniels, 1965; Chan *et al.*, 1975). This discovery led to a systematic investigation of the phototoxic properties of polyacetylenes and their thiophene derivatives from the Compositae by Towers and his associates (e.g., Camm *et al.*, 1975; Towers, 1980; Towers and Wat, 1978; Towers *et al.*, 1977; Wat *et al.*, 1980).

It is now well established that many polyacetylenes, notably α-terthienyl and phenylheptatriyne (Table I), are toxic to biological systems in the presence of UV-A (320-400nm) radiation. In addition to bacteria and fungi (Arnason *et al.*,1980; DiCosmo *et al.*, 1982), these compounds kill human fibroblasts and erythrocytes (Wat *et al*., 1977; MacRae *et al*., 1980b; Towers *et al*., 1979), cercaria (Graham *et al*., 1980), adult nematodes, insect larvae and eggs (Arnason *et al*., 1981; Kagan and Chan, 1983; Wat *et al*., 1981) and deactivate viruses (Warren *et al*., 1980; Hudson *et al*., 1982), but they are not genotoxic (MacRae *et al*., 1980a).

Unlike the linear furanocoumarins, whose phototoxic effects can be explained by the photo-induced modification of DNA (Song and Tapley, 1979), polyacetylenes act on cell membranes. Specifically, α-terthienyl acts as a typical Type II photodynamic sensitizer, requiring oxygen for its activity while the photosensitization of *E. coli* cells and erythrocytes by phenylheptatriyne occurred under both aerobic and anaerobic conditions (Wat *et al*., 1980; Arnason *et al*., 1981; McLachlan *et al*., 1984).

Many details are known about polyacetylenes and their *in vitro* effects and, although there is considerable speculation about their putative *in vivo* functions, at present, no obvious physiological role can be allocated to polyacetylenes in the organisms which produce them. This does not preclude practical application of their potent biocidal properties. Polyacetylenes are notoriously unstable and decompose rapidly in aqueous solution and in light (e.g., Anchel *et al*., 1950; Celmer and Solomon, 1953; Bohlmann *et al*., 1973; Towers, 1980). This rapid biodegradability may certainly be exploited to advantage in the search for effective and environmentally nontoxic biological control agents.

The purpose of this study is to explore various aspects of polyacetylenes in one group of plants, Hawaiian *Bidens*, in order to establish preliminary information on their

1. occurrence and evolutionary significance,
2. biosynthetic pathways, and
3. antibiotic properties.

Such preliminary data are necessary in order to identify those hypotheses which will lead to a rapid growth of knowledge concerning these chemicals. As well, this information will be useful in determining their potential usefulness and define the limits of manipulation for human utilization.

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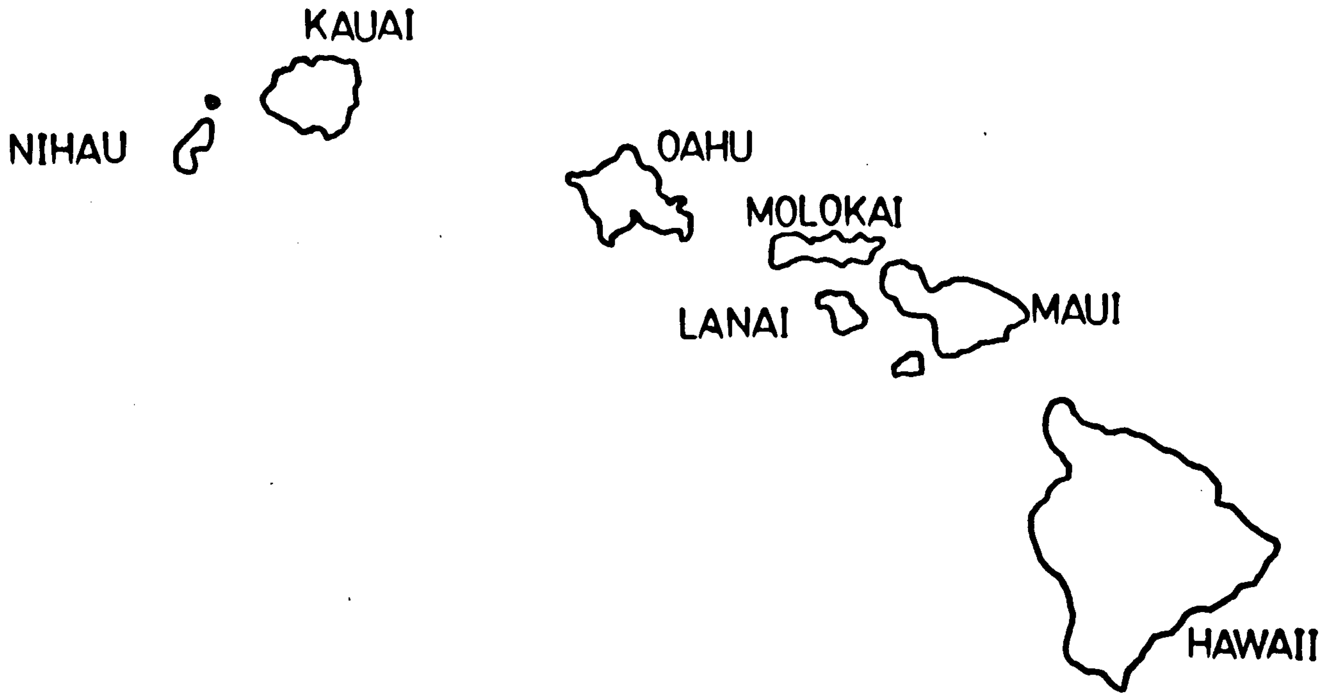
II. POLYACETYLENES IN HAWAIIAN *BIDENS*

A. INTRODUCTION

The Hawaiian Islands are usually considered to be the most isolated archipelago on earth. They lie virtually alone in the North Pacific, separated from the nearest high oceanic islands such as the Marquesas by 3200km, and from the North American coast by 4000km of ocean (Carlquist, 1970). All are giant submarine volcanoes that arose from an apparently stationary hot spot beneath the Pacific Plate which has been moving in a northwesterly direction for tens of millions of years. The island chain stretches 2500km from northwest to southeast across the Pacific, the older Western Leeward Islands having been reduced by erosion to shoals, islets and atolls, while the main eastern group, the Windward Islands, are mountainous and geologically young. The major islands range in age from 5.6 million years for Kauai to less than 1.0 million years for Hawaii (Figure 1). Geological evidence indicates that the Hawaiian chain has never been connected to a continental land mass (Stearns, 1966).

The ancestors of all indigenous Hawaiian plants and animals arrived by accidental long-distance dispersal. The rainfall, soil and temperature conditions of the Hawaiian Islands make them exceptionally inviting fields for occupation by many groups of organisms. In addition, there is considerable variation in microclimate on each island



THE MAJOR HAWAIIAN ISLANDS

FIGURE 1. THE HAWAIIAN ISLANDS

because of the mountainous topography. Upon arrival, immigrant species faced a new, physically diverse environment with a relative absence of competition, conditions ideal for adaptive radiation (Carlquist, 1966a; 1966b; 1967; 1970). Adaptive radiation is the evolutionary diversification of one ancestral lineage into numerous species adapted to life in a variety of habitats. Many groups of organisms such as the honeycreepers, flies (*Drosophila* Fallén), rutaceous plants (*Pelea* A. Gray) and composites (*Lipochaeta* DC.) have undergone spectacular radiation in the Hawaiian Islands. The genus *Bidens* L. (Asteraceae), commonly known as beggarticks or Spanish needles in North America and ko'oko'olau in Hawaii, has evolved from a single ancestral species into numerous taxa which exhibit greater morphological and ecological diversity than species of *Bidens* found elsewhere in the world (Ganders and Nagata, 1983a; 1984).

The Hawaiian species of *Bidens* occur in habitats that range from arid to semi-arid lava flows with less than 0.3m of rainfall per year, to dense rainforest and montane bogs with annual precipitation exceeding 7.0m, and through elevations extending from sea level to over 2200m. They have diversified in growth habit (from small trees with woody trunks over 2m tall to tall shrubs to erect and prostrate herbaceous forms), leaf shape (from simple to compound to highly dissected), flower head size and shape, achene size and shape (from flat and straight to tightly coiled), presence and type of dispersal mechanism (awns of various lengths and shapes, pubescence, and presence or absence of wings), as well as in ecological tolerances. Differences between species in all these characters are maintained under standard growing conditions, indicating that they are under strong genetic control (Helenurm and Ganders, 1985).

Surprisingly, however, all Hawaiian *Bidens* are completely interfertile (Ganders and Nagata, 1984). This suggests that adaptive radiation in morphology and ecological tolerance has occurred without the evolution of physiological or genetic interspecific isolating mechanisms (Gillett and Lim, 1970; Gillett, 1975; Ganders and Nagata, 1983a; 1984). The different species of Hawaiian *Bidens* are as similar genetically at isozyme loci as are populations of a single species in most plants. They exhibit little differentiation in isozymes of primary metabolic processess, and genetic distances among populations based on isozyme loci show little correlation with morphological differences or taxonomic classification (Helenurm and Ganders, 1985). This disparity between the evolution of morphological and biochemical characters makes it of interest to determine whether or not there has been evolutionary divergence in secondary metabolites in these species.

In this study, the polyacetylenes from leaves and roots of all the endemic Hawaiian species of *Bidens* and all but two of the subspecies were isolated and identified. The primary objectives were to determine the extent of evolutionary differentiation of polyacetylenes in Hawaiian *Bidens* and to see whether they are useful taxonomic characters in the group. The possible relationship of polyacetylene distribution to the biology of Hawaiian *Bidens* is also considered.

Sherff (1943) produced a worldwide taxonomic revision of the genus *Bidens* based on a study of herbarium material. In this and subsequent publications he recognized 43 species and more than 20 infraspecific taxa endemic to the Hawaiian Islands. He had no information, however, on the extent of environmentally determined variation in these plants. Ganders and Nagata (1983a; 1984) have reduced these to 19 species and 8 subspecies. Their classification is followed in this thesis.

Since all the species of Hawaiian *Bidens* are interfertile, interspecific hybrids were relatively easy to obtain experimentally. Several of these hybrids, as well as their F_2 offspring, were examined for their polyacetylenes. The purpose of this portion of the study was to determine the degree of complexity of polyacetylene inheritance.

B. MATERIALS AND METHODS

PLANT MATERIAL

Plants from 54 populations representing 19 species and six subspecies of endemic Hawaiian *Bidens* were examined for polyacetylenes in leaves and roots (Table II). This includes all endemic taxa recognized by Ganders and Nagata (1984) except *B. campylotheca* Schz. Bip. ssp. *waihoiensis* St.John and *B. hillebrandiana* (Drake del Cast.) Deg. ex Sherff ssp. *hillebrandiana* . Localities for all populations are shown in Figures 1 to 6. Voucher specimens are deposited at the University of British Columbia (UBC), and duplicates of most are also at the Harold Lyon Arboretum, Honolulu (HLA). F_1 and F_2 hybrids were synthesized by F.R.Ganders and all plants were grown from seeds or cuttings in greenhouses at the University of British Columbia under natural light, and leaves and roots of greenhouse plants harvested for analysis.

ISOLATION AND IDENTIFICATION OF POLYACETYLENES

Fresh leaves and roots were extracted with methanol (MeOH) (1g to 10ml ratio), ground and filtered. The filtrate was diluted 1:1 with distilled water and extracted twice with equal volumes of light petroleum ether(PE) (30-60°C). The combined PE fractions were dried with anhydrous Na_2SO_4. Solvent volume was reduced to 3ml for spectral analysis. UV spectra were recorded in spectral grade PE using either a

TABLE II. *BIDENS* TAXA EXAMINED FOR POLYACETYLENES

1. *Bidens amplectens* Sherff
2. *B. asymmetrica* (Lévl.) Sherff
3. *B. campylotheca* Schz. Bip. ssp. *campylotheca*
3a. *B. campylotheca* ssp. *pentamera* (Sherff) Nagata & Ganders
4. *B. cervicata* Sherff
5. *B. conjuncta* Sherff
6. *B. cosmoides* (Gray) Sherff
7. *B. forbesii* Sherff ssp. *forbesii*
7b. *B. forbesii* ssp. *kahiliensis* Ganders & Nagata
8. *B. hawaiensis* Gray
9. *B. hillebrandiana* ssp. *polycephala* Nagata & Ganders
10. *B. macrocarpa* (Gray) Sherff
11. *B. mauiensis* (Gray) Sherff
12. *B. menziesii* ssp. *menziesii* (Gray) Sherff
12a. *B. menziesii* ssp. *filiformis* (Sherff) Ganders & Nagata
13. *B. micrantha* Gaud. ssp. *micrantha*
13b. *B. micrantha* ssp. *ctenophylla* (Sherff) Nagata & Ganders
13a. *B. micrantha* ssp. *kalealaha* Ganders & Nagata
14. *B. molokaiensis* (Hillebr.) Sherff
15. *B. populifolia* Sherff
16. *B. sandvicensis* Less. ssp. *sandvicensis*
16a. *B. sandvicensis* ssp. *confusa* Nagata & Ganders
*17. *B. torta* Sherff
18. *B. valida* Sherff
19. *B. wiebkei* Sherff

* 17A (B18, B19); 17B (B36-B41); 17C (B55, B56); 17D (B110)

FIGURE 2. LOCALITIES OF KAUAI *BIDENS* POPULATIONS SAMPLED

B. cervicata: B8, B83; *B. cosmoides*: B9; *B. forbesii* ssp. *forbesii*: B12, B13, B14, B74, B101, B124: *B. forbesii* ssp. *kahiliensis*: B71, B134: *B. sandvicensis* ssp. *sandvicensis*: B112; *B. sandvicensis* ssp. *confusa*: B33, B34; *B. valida*: B54, B131, B132.

KAUAI

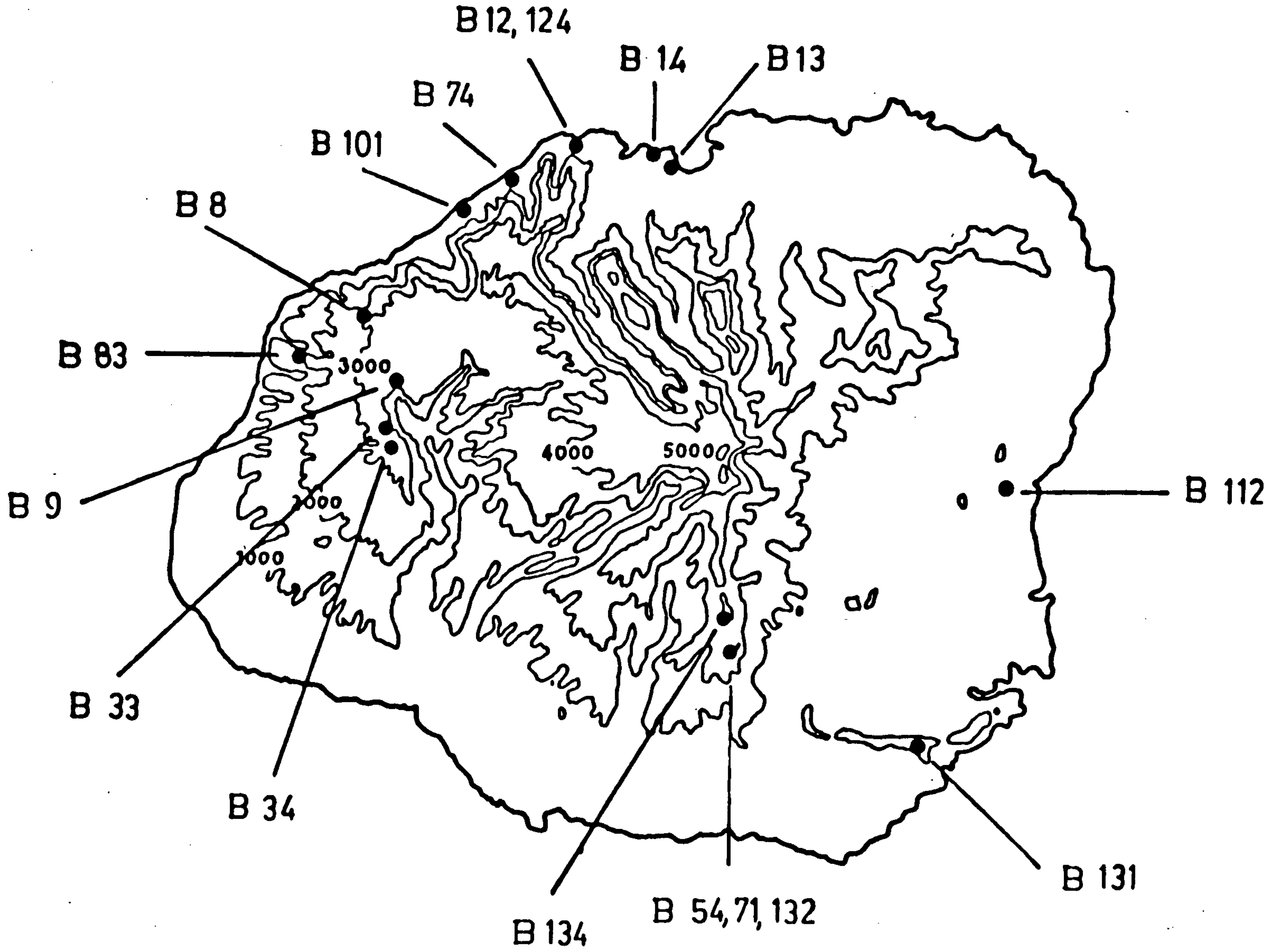

B 12, 124
B 14
B 13
B 74
B 101
B 8
B 83
3000
B 9
4000
5000
B 112
2000
1000
B 33
B 34
B 134
B 54, 71, 132
B 131

FIGURE 3. LOCALITIES OF OAHU *BIDENS* POPULATIONS SAMPLED

B. amplectens: B1; *B. asymmetrica*: B4; B211; *B. campylotheca* ssp. *campylotheca*: B195; *B. cervicata*: B88; *B. macrocarpa*: B22; B23; *B. molokaiensis*: B11; *B. populifolia*: B42; *B. sandvicensis* ssp. *sandvicensis*: B5; B6; B7; B20; B35; B43-B47; *B. torta*: B18, B19 (A), B36-B41 (B), B55, B56 (C), B110 (D).

OAHU

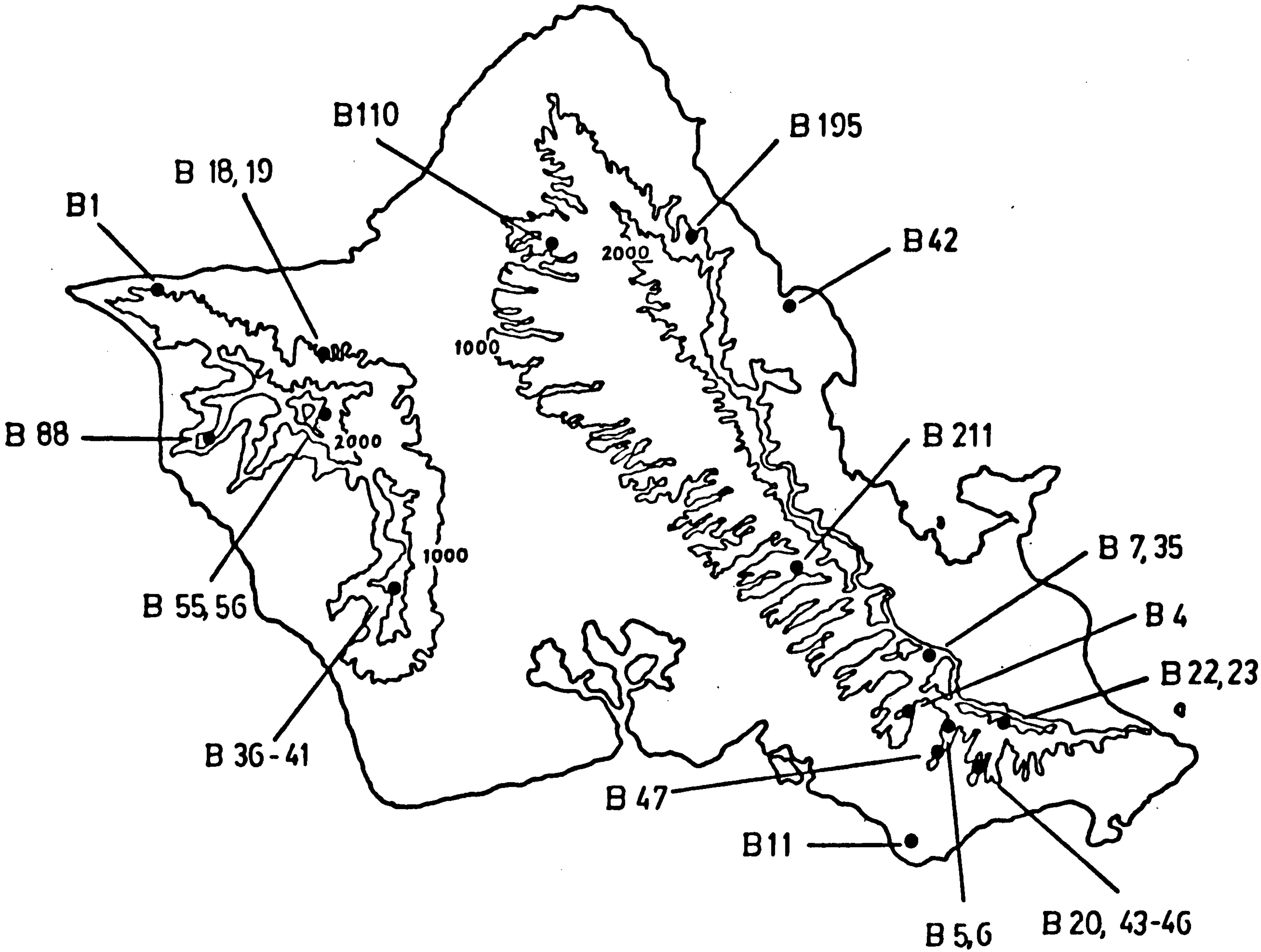

B110
B 18, 19
B1
B 195
B 42
2000
1000
B 88
2000
1000
B 55, 56
B 36 - 41
B 211
B 7, 35
B 4
B 22, 23
B 47
B 11
B 5, 6
B 20, 43-46

FIGURE 4. LOCALITIES OF MAUI *BIDENS* POPULATIONS SAMPLED

B. campylotheca ssp. *pentamera*: B114; *B. conjuncta*: B60-B63; *B. hillebrandiana* ssp. *polycephala*: B67, B68; *B. mauiensis*: B10, B27(cultivated), B28, B128; *B. menziesii* ssp. *menziesii*: B31, B84; *B. micrantha* ssp. *micrantha*: B24, B25, B78, B79; *B. micrantha* ssp. *kalealaha*: B125.

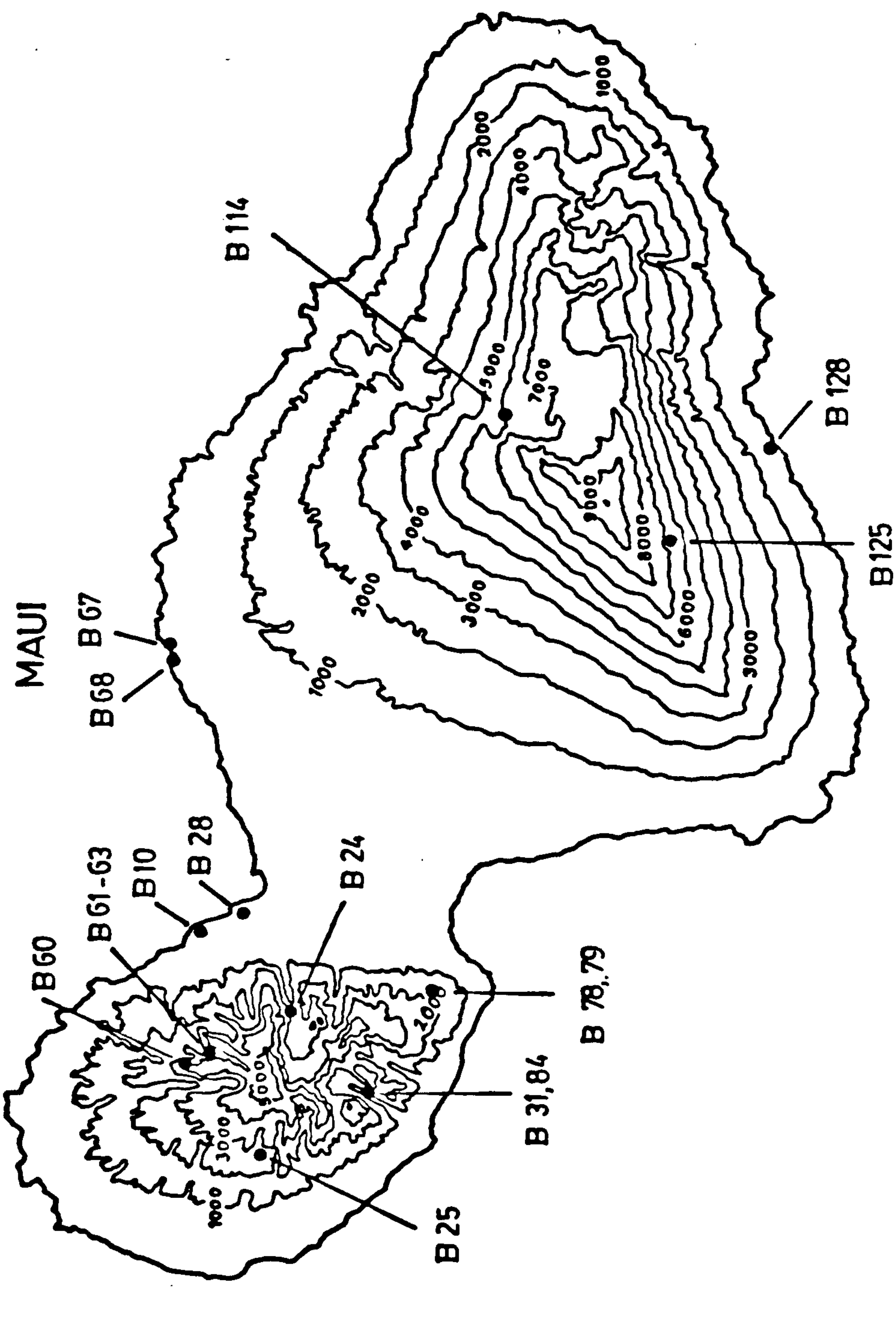

MAUI
B 114
B 128
B 125
B 67
B 68
B 28
B 10
B 61-63
B 60
B 24
B 78,79
B 31,84
B 25
1000
2000
3000
4000
5000
6000
7000
8000
9000

FIGURE 5. LOCALITIES OF MOLOKAI *BIDENS* POPULATIONS SAMPLED. *B. molokaiensis*: B72, B73; *B. weibkei*: B260.

MOLOKAI

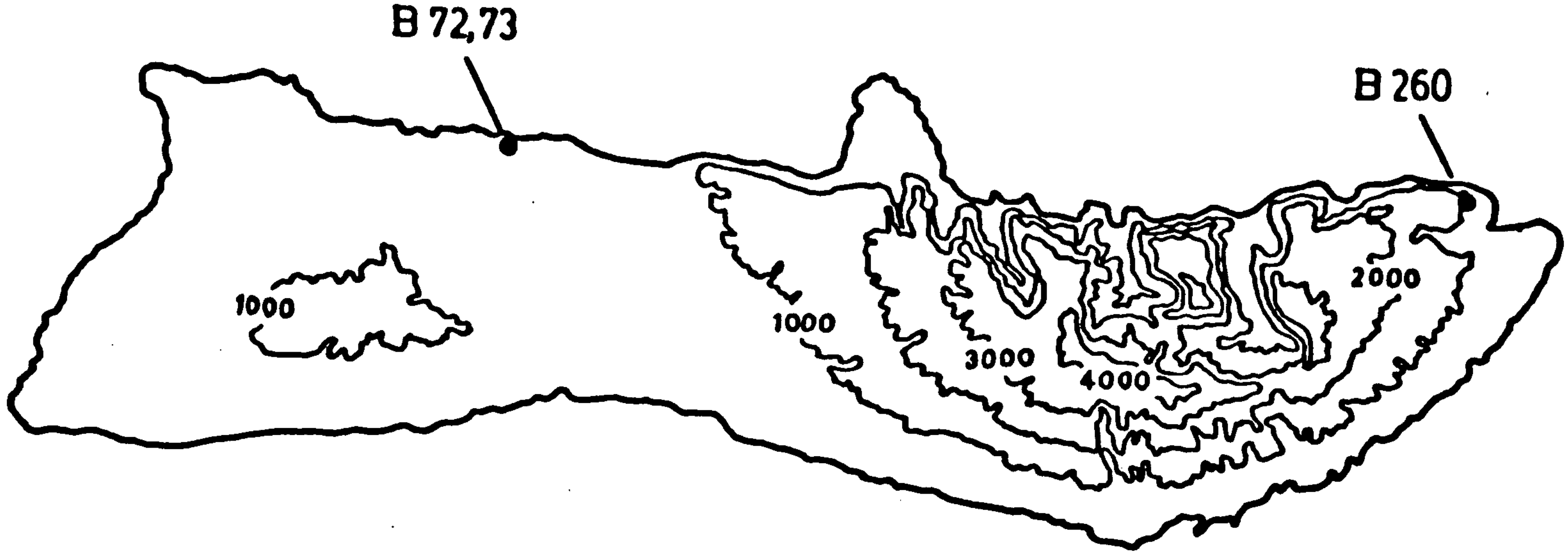
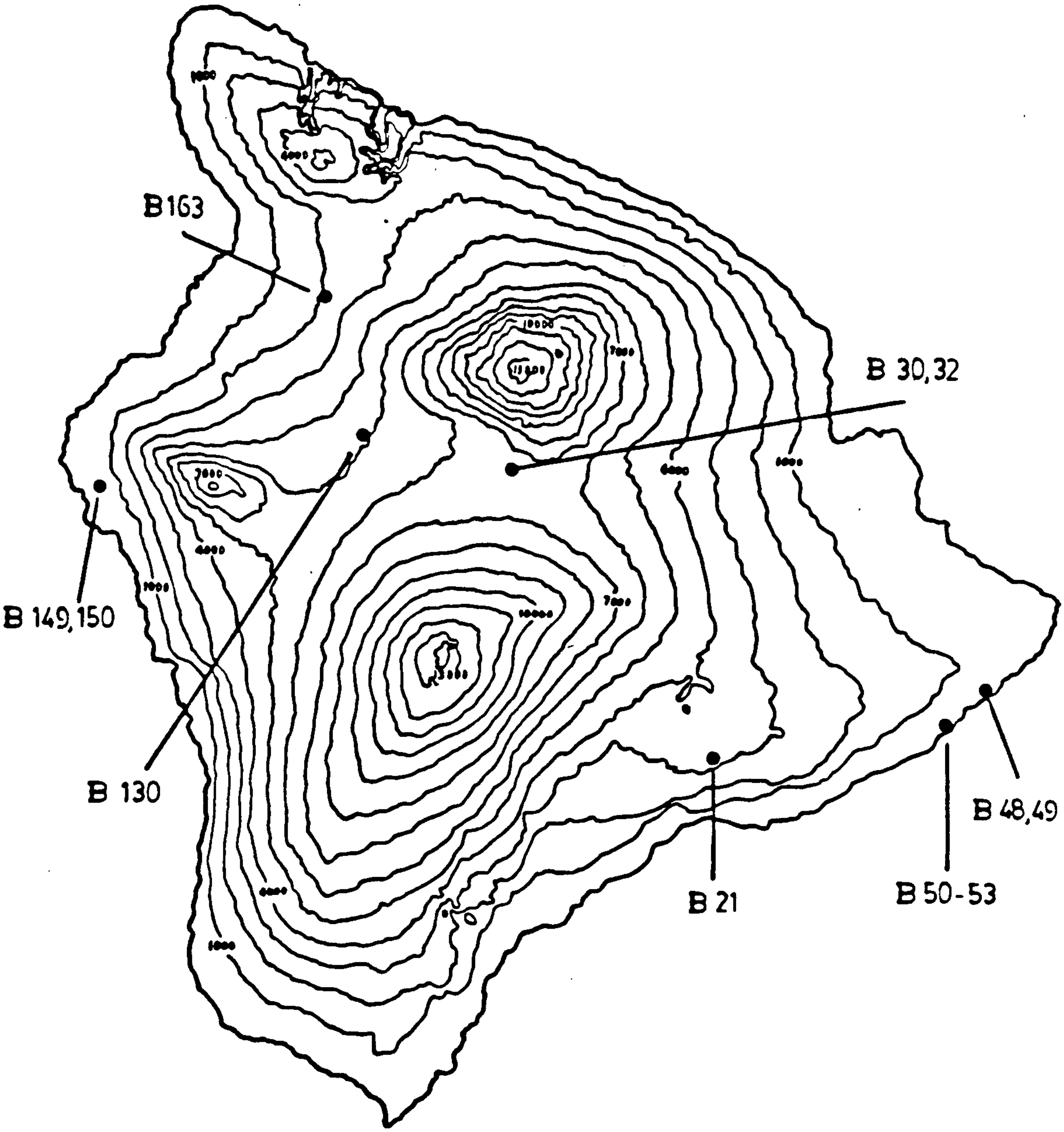


FIGURE 6. LOCALITIES OF HAWAII *BIDENS* POPULATIONS SAMPLED

B. hawaiensis: B21, B48-B53; *B. menziesii* ssp. *filiformis*: B30, B32, B130, B163; *B. micrantha* ssp. *ctenophylla*: B149, B150.

HAWAII



Pye-Unicam SP8-100 UV/VIS or a Unicam SP800A UV/VIS spectrophotometer. Samples were evaporated to dryness under nitrogen and resuspended in 1ml spectral grade MeOH for storage at -20°C. Concentrated samples were injected into a Finnigan 1020 Automated GC/MS and the mass spectra of compounds in the PE fraction, including polyacetylenes, recorded. Chromatographic separation of compounds was carried out with a SE-54 30m x 0.25mm capillary column using a temperature gradient of 10°/min from 150°C to 250°C, and helium as a carrier gas.

Polyacetylenes were separated on analytical silica gel sheets containing a fluorescent indicator (SG-UV254 and SG N-UV254). PE (30-60°C) with increasing percentages of diethyl ether(DE), was used to effect separation of the compounds in each extract according to the methods decribed by Lam *et al*. (1968) and Wrang and Lam (1975). Column chromatography on silica gel 60 and a chromatotron (Harrison Research) using silica gel PF-254 with $CaSO_4 \cdot 1/2H_2O$ plates were used for larger scale separations. Columns and plates were developed with PE (30-60°C) followed by increasing amounts of DE to elute more polar compounds. The solvent systems used for all separations were of the following proportions of PE to DE :19:1; 9:1; 17:3; 8:2; 7:3; 13:7; 5:5. A small aliquot of concentrated acetic acid was added to the developing tank for thin layer chromatography. In all cases, compounds **1** to **18** were eluted by PE/DE 13:7.

Individual acetylenes were identified by comparison of UV and mass spectra with those of known compounds. Extraction, isolation and identification procedures were carried out in dim light at 0°C or lower.

Individuals from one to five populations of each taxon were examined over a period of 18 months. Leaves from individual plants were analyzed for polyacetylenes three to six times throughout this period. Roots were analyzed twice. F_1 individuals were analyzed three times over 12 months and F_2 populations sampled once. This precluded large scale extractions for specific compounds.

C. RESULTS

POLYACETYLENES IN *BIDENS* TAXA

Leaves and roots from 19 species and six subspecies of *Bidens* from Kauai, Oahu, Maui, Molokai and Hawaii were analyzed for polyacetylenes (Table II, Figures 2 to 6). Two taxa (*B. campylotheca* Schz. Bip. ssp. *waihoiensis* St. John and *B. hillebrandiana* (Drake del Cast.) Deg. ex Sherff ssp. *hillebrandiana*) were not available for analysis. Compounds 1 to 18 were isolated chromatographically and identified on the basis of UV and mass spectra (Tables III, IV) and their distribution among the species recorded (Tables V, VI).

Although acetylenes were found in all the root samples examined, they were absent from the leaves of 13 of the taxa (Table V). Repeated sampling of greenhouse populations over a period of 18 months revealed no qualitative variation in polyacetylene production with changes in season or reproductive state of the plants. This is in contrast to *Dahlia* , where considerable variation in polyacetylene content and composition were encountered within the species in consecutive seasons, and in the same season in plants growing in different locations (Chin *et al*., 1970; Lam *et al*.,1968). Many acetylenes are known to be photoactive (Towers *et al*., 1977; Towers, 1980), and the crude light petroleum fractions of leaf and root extracts were tested for phototoxicity against nine species of fungi and bacteria using the method of Daniels (1965). While the root samples

TABLE III. POLYACETYLENES FROM HAWAIIAN *BIDENS*

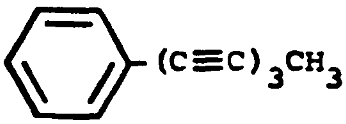
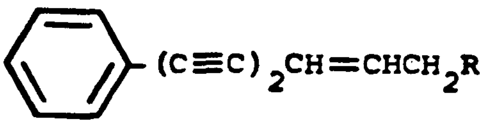
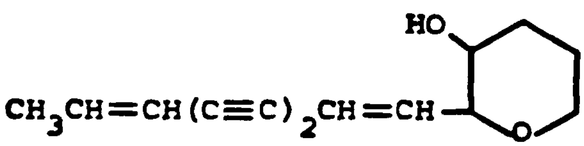
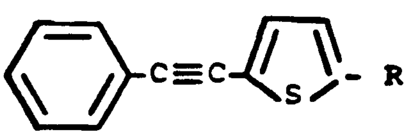
$CH_2{=}CH(C{\equiv}C)_4CH{=}CHCH_2R$	R = H	1
	R = $OCOCH_3$	2
$CH_2{=}CH(C{\equiv}C)_5CH_3$		3
		4
	R = H	5
	R = OH	6
	R = $OCOCH_3$	7
$CH_3CH{=}CH(C{\equiv}C)_2(CH{=}CH)_2(CH_2)_4CH{=}CH_2$		8
$CH_3(C{\equiv}C)_3(CH{=}CH)_2(CH_2)_4CH{=}CH_2$		9
$CH_3CH{=}CH(C{\equiv}C)_2CH_2CH{=}CH(CH_2)_5CH{=}CH_2$		10
$CH_2{=}CHCH{=}CH(C{\equiv}C)_3CH{=}CHCH_2R$	R = H	11
	R = $OCOCH_3$	12
		13
	R = CH_3	14
	R = CH_2OH	15
	R = CHO	16
	R = CH_2OCOCH_3	17
$CH_3CH{=}CH(C{\equiv}C)_3CH{=}CHCHOHCH_2OH$		18

TABLE IV. POLYACETYLENES FROM HAWAIIAN *BIDENS*

1,2	trideca-1,11 diene-3,5,7,9 tetrayne
3	trideca-1 ene-3,5,7,9,11 pentayne
4	1-phenylhepta-1,3,5 triyne
5,6,7	1-phenylhepta-1,3 diyne-5 ene
8	heptadeca-2,7,9,16 tetraene-4,6 triyne
9	heptadeca-8,10,16 triene-2,4,6 triyne
10	heptadeca-2,9,16 triene -4,6 diyne
11,12	trideca-1,3,11 triene-5,7,9 triyne
13	tetrahydro-2 1,7 diene-3,5 diynyl pyran-3-ol
14-17	2- 2-phenylethyne-1 yl -5 methyl thiophene
18	trideca-3,11 diene-5,7,9 triyne-1,2 diol

TABLE V. POLYACETYLENES IN THE LEAVES OF HAWAIIAN *BIDENS*

	Compounds											
Bidens	1	2	3	4	5	7	8	9	10	11	12	18
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	+	-	-	-	-	-	+	-	+	-	-	+
3a	+	-	-	-	+	+	+	-	+	-	-	-
4	+	-	-	-	+	+	-	-	-	-	-	-
5	-	-	-	-	-	-	+	-	-	-	-	-
6	+	-	+	-	+	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
7a	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	+
9	+	+	-	-	-	-	-	-	-	+	+	-
10	+	-	-	-	-	-	+	-	+	-	-	+
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	+	-	+	-	-	-
12a	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
13a	-	-	-	-	-	-	-	-	-	-	-	-
13b	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
16a	-	-	-	-	+	-	-	-	-	-	-	-
17A	-	-	-	+	-	-	-	-	-	-	-	-
17B	-	-	-	-	+	-	-	-	-	-	-	-
17C	-	-	-	-	-	-	+	+	-	-	-	-
17D	-	-	-	-	+	-	-	-	-	-	-	-
18	+	-	-	-	+	-	+	-	-	+	+	-
19	-	-	-	-	-	-	-	-	-	-	-	-

TABLE VI. POLYACETYLENES IN THE ROOTS OF HAWAIIAN *BIDENS*

	Compounds													
Bidens	1	2	5	6	7	8	10	11	12	13	14	15	16	17
1	+	-	-	-	-	-	-	-	-	-	-	-	-	+
2	+	-	-	-	-	-	+	+	+	+	-	-	-	+
3	+	+	-	-	-	-	+	-	-	+	-	-	-	+
3a	+	+	-	-	-	-	+	-	-	-	+	-	-	+
4	+	+	-	-	-	-	+	-	-	-	-	+	-	+
5	+	-	+	-	+	-	+	-	-	+	-	-	-	+
6	+	+	+	-	+	+	+	-	-	-	-	-	+	+
7	+	-	-	-	-	-	+	-	-	+	-	-	-	+
7a	+	+	+	-	-	-	+	-	+	+	-	-	+	+
8	+	-	-	-	-	-	+	-	+	-	-	+	-	+
9	+	+	-	-	-	-	-	-	+	-	-	-	+	+
10	+	+	-	-	-	+	+	-	-	-	-	-	+	+
11	+	-	-	-	-	-	+	-	+	+	-	-	-	+
12	+	-	+	+	+	+	+	-	-	-	-	+	-	+
12a	+	-	-	-	+	-	+	-	-	-	+	-	+	+
13	+	+	-	-	-	-	-	-	+	-	+	+	+	+
13a	+	+	-	-	-	-	+	-	+	+	-	+	+	+
13b	+	-	-	-	-	-	-	-	-	-	-	-	-	+
14	+	-	-	-	-	-	-	-	-	-	-	-	-	+
15	+	+	-	-	-	-	+	-	-	-	-	-	-	+
16	+	+	-	-	+	-	-	-	-	-	-	-	-	+
16a	+	-	-	-	+	-	-	-	-	-	-	-	-	+
17A	-	-	-	-	+	-	-	-	-	-	-	-	+	+
17B	+	-	+	-	+	-	+	-	+	+	-	-	+	+
17C	+	-	-	-	-	-	+	-	+	-	-	+	-	+
17D	+	+	-	-	-	-	+	-	-	-	-	-	-	+
18	+	-	-	-	-	-	+	-	-	-	-	-	-	+
19	+	+	-	-	-	-	-	-	-	-	-	-	-	+

were found to be consistently phototoxic, only leaf extracts containing acetylenes were lethal to the microorganisms in the presence of near UV radiation (320-400nm) (see Chapter IV). The absence of polyacetylenes in the leaves of at least two other *Bidens* species has been previously noted (Bohlmann *et al.*,1973), so this feature is not unique to Hawaiian *Bidens*. However, it does lead to interesting speculation about the possible biological significance of the compounds in question.

The polyacetylenes of Hawaiian *Bidens* include eleven C_{13} hydrocarbons, aromatic and thiophenic derivatives, a C_{14} tetrahydropyran and three C_{17} hydrocarbons (Table III, IV). Compounds **1** to **18** can be derived from oleic acid by a series of dehydrogenations, oxidations and reductions (Figure 7). Except for **14** to **18**, they have previously been reported in North American and European *Bidens*, *Coreopsis* and/or *Dahlia* (Bohlmann *et al.*,1973). Compound **16** occurs in the leaves of *C.grandiflora* Hogg ex Sweet (Bohlmann *et al.*,1966) while **18** ('safynol') has been reported in *Carthamus tinctorius* L. (Bohlmann *et al.*,1966) and two species of *Centaurea* (Anderson *et al.*,1977; Bohlmann *et al.*,1958). Naturally-occurring **14**, **15** and **17** have not been isolated before although the isomers **19** and **20** (Figure 8), are found in *Coreopsis grandiflora* and *C.nuecensis* F. Heller, respectively (Bohlmann and Zdero,1968; Lam *et al.*,1968; Sørensen and Sørensen, 1958). Although the presence of the acetate (**17**) presumes the existence of the precursors (**14**,

FIGURE 7. BIOGENETIC RELATIONSHIPS OF POLYACETYLENES FROM HAWAIIAN *BIDENS*

Compounds 1 to 18 can be derived from oleic acid via the intermediate dehydrocrepenynic acid by a series of transformations which include dehydrogenations (*), α-oxidations (α), β-oxidations (β), and the addition of H_2S (adapted from Bohlmann *et al.*, 1978).

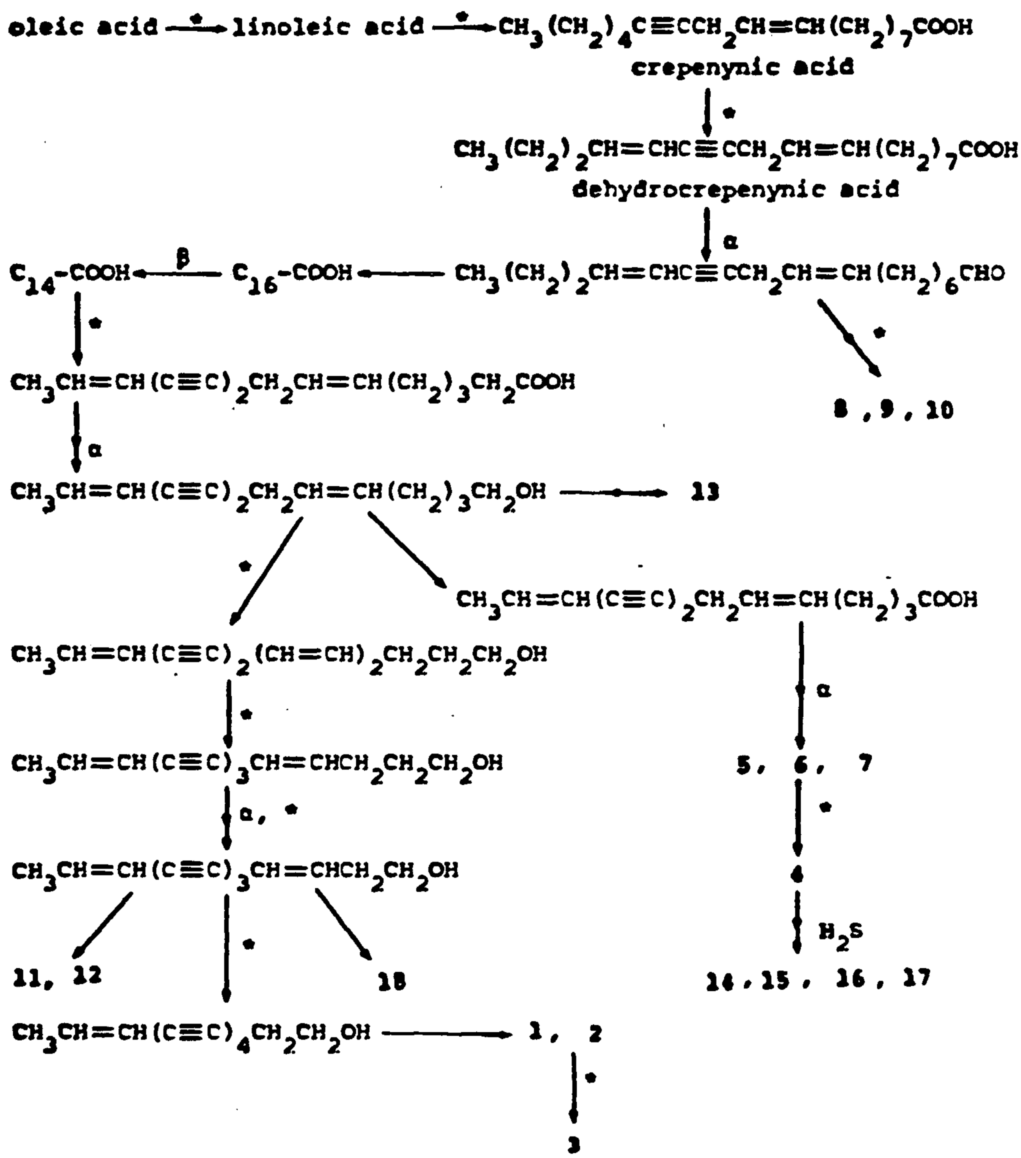
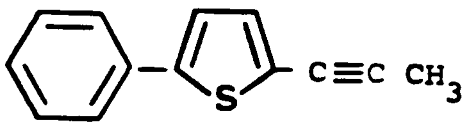
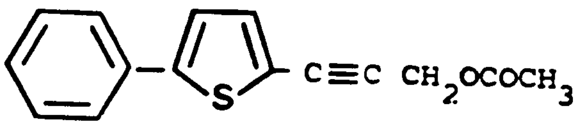

oleic acid
linoleic acid
CH3(CH2)4C≡CCH2CH=CH(CH2)7COOH
crepenynic acid
CH3(CH2)2CH=CHC≡CCH2CH=CH(CH2)7COOH
dehydrocrepenynic acid
C14-COOH
C16-COOH
CH3(CH2)2CH=CHC≡CCH2CH=CH(CH2)6CHO
8, 9, 10
CH3CH=CH(C≡C)2CH2CH=CH(CH2)3CH2COOH
CH3CH=CH(C≡C)2CH2CH=CH(CH2)3CH2OH
13
CH3CH=CH(C≡C)2CH2CH=CH(CH2)3COOH
CH3CH=CH(C≡C)2(CH=CH)2CH2CH2CH2OH
5, 6, 7
CH3CH=CH(C≡C)3CH=CHCH2CH2CH2OH
4
H2S
CH3CH=CH(C≡C)3CH=CHCH2CH2OH
11, 12
18
14, 15, 16, 17
CH3CH=CH(C≡C)4CH2CH2OH
1, 2
3

FIGURE 8. PHENYLTHIOPHENES FROM *COREOPSIS*



19



20

15 and 16), these were not detected in many of the root extracts. Compound 17 is ubiquitous in *Bidens* roots, and although it may not serve as a taxonomic marker, its unusual structure suggests that it may have phototoxic and other interesting biological properties (Towers, 1979).

Compound 3('pentayne-ene'), which is common in the Asteraceae (Bohlmann *et al.*,1973), was found in trace quantities in the leaves of only one of the species, *B.cosmoides* . This may be due to its extreme instability or to low concentrations. Since stringent precautions were taken to prevent polyacetylene degradation during laboratory workup however, and since the compound has a relatively high extinction coefficient ($\epsilon=10^5$), it would appear that the pentayne-ene does not accumulate in most species of Hawaiian *Bidens*. The other highly conjugated acetylene hydrocarbon 1 ('ene-tetrayne-ene'), was detected spectrophotometrically in many leaves and in all crude root extracts except that of *B.torta* A (17A).

POLYACETYLENES IN *BIDENS* HYBRIDS

Leaves from 21 *Bidens* hybrids were analyzed for polyacetylenes. Compounds were isolated chromatographically and identified on the basis of UV and mass spectra, and their distribution recorded in Tables VII to XI. The leaves of F_2 populations from seeds of two selfed F_1 individuals were also examined (five individuals of selfed *B.*

TABLE VII *BIDENS* HYBRIDS EXAMINED FOR POLYACETYLENES

1. *B. sandvicensis* ssp. *sandvicensis* X *B. molokaiensis*
2. *B. sandvicensis* ssp. *sandvicensis* X *B. micrantha* ssp. *micrantha*
3. *B. valida* X *B. molokaiensis*
4. *B. molokaiensis* X *B. macrocarpa*
5. *B. molokaiensis* X *B. cosmoides*
6. *B. forbesii* ssp. *forbesii* X *B. cosmoides*
7. *B. menziesii* ssp. *filiformis* X *B. cosmoides*
8. *B. micrantha* ssp. *micrantha* X *B. valida*
9. *B. sandvicensis* ssp. *sandvicensis* X *B. valida*
10. *B. menziesii* ssp. *filiformis* X *B. hawaiensis*
11. *B. micrantha* ssp. *micrantha* X *B. hawaiensis*
12. *B. sandvicensis* ssp. *confusa* X. *B. sandvicensis*
13. *B. sandvicensis* ssp. *confusa* X *B. mauiensis*
14. *B. forbesii* ssp. *forbesii* X *B. torta* 17A
15. *B. torta* 17A X *B. micrantha* ssp. *micrantha*
16. *B. populifolia* X *B. torta* 17C
17. *B. torta* 17B X *B. hawaiensis*
18. *B. torta* 17A X *B. valida*
19. *B. valida* X *B. macrocarpa*
20. *B. carvicata* X *B. cosmoides*
21. *B. cervicata* X *B. macrocarpa*

TABLE VIII. POLYACETYLENES FROM *BIDENS* HYBRIDS

Bidens F_1	Compounds 1	2	3	4	5	7	8	9	10	11	12	18
Type A Cross												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
Type B Cross												
3	+	-	-	-	+	-	-	-	-	-	+	-
4	+	-	-	+	+	-	+	-	+	-	-	-
5	+	-	+	+	+	-	+	-	+	-	-	-
6	+	-	+	+	+	-	+	-	+	-	-	-
7	+	-	+	+	+	-	+	-	+	-	-	-
8	-	-	-	-	+	-	-	-	-	-	-	-
9	-	-	-	-	+	-	-	-	-	-	-	-
10	-	+	-	+	+	-	-	-	-	-	+	+
11	-	-	-	-	+	-	-	-	-	-	+	+
12	-	+	-	-	+	-	-	-	-	-	-	-
13	-	+	-	-	+	-	-	-	-	-	-	-
14	-	-	-	+	-	-	-	-	-	-	-	-
15	-	-	-	-	+	+	-	-	-	-	-	-
16	-	-	-	-	-	-	+	+	-	-	-	-
Type C Cross												
17	-	-	-	-	+	-	-	-	-	-	+	+
18	+	-	-	+	+	-	-	-	-	+	+	-
19	+	-	-	-	+	+	-	-	-	-	+	-
20	+	-	+	+	+	-	+	-	+	-	-	-
21	+	-	+	-	+	-	+	+	+	-	-	-

TABLE IX. POLYACETYLENES IN *B. HAWAIENSIS* HYBRIDS

	Compounds								
Bidens F_1	1	2	3	4	5	7	11	12	18
Type B Cross									
10	-	*+	-	*+	+	-	-	*+	+
11	-	-	-	-	+	-	-	*+	+
Type C Cross									
17	-	-	-	-	+	-	-	*+	+

*absent in parents

TABLE X. POLYACETYLENES IN *B. COSMOIDES* HYBRIDS

	Compounds								
Bidens F_1	1	2	3	4	5	7	8	9	10
Type B Cross									
5	+	-	+	*+	+	-	*+	-	*+
6	+	-	+	*+	+	-	+	-	*+
7	+	-	+	*+	+	-	*+	-	*+
Type C Cross									
20	+	-	+	*+	+	-	*+	-	*+

*absent in parents

TABLE XI. POLYACETYLENES IN *B. MACROCARPA* HYBRIDS

Bidens F_1	Compounds 1	2	3	4	5	7	8	9	10	11	12	18
Type B Cross												
4	+	–	–	*+	+	–	+	–	+	–	–	▲–
Type C Cross												
19	+	–	–	–	*+	+	▲–	–	▲–	–	+	▲–
21	+	–	*+	–	+	–	+	*+	+	–	–	▲–

*absent in parents

▲ present in parents but absent in F_1

sandvicensis ssp. *confusa* X *B. mauiensis*, B185s, and 22 individuals of *B. sandvicensis* ssp. *confusa* X *B. sandvicensis* ssp. *sandvicensis*, B194s, (Table XII).

Type A crosses between two species which do not produce leaf acetylenes result in F_1 individuals without acetylenes (Table VIII). Crosses between species which produce leaf acetylenes and those which do not (Type B) result in hybrids which synthesize leaf acetylenes although the compounds are not always identical to the arrays present in the parents (Table VIII to XI). In most crosses of this category, the F_1 progeny did not produce any compounds absent from the leaves of the parents. Nevertheless, in crosses with *B. hawaiensis* , compound **12** is expressed in the leaves of the hybrids (Table IX). *Bidens hawaiensis* leaves characteristically produce compound **18** (Tables III, IV), one which is closely related to compound **12** in the proposed biogenetic scheme shown in Figure 7. Compound **12** also occurs in the roots of *B. hawaiensis*. Compound **4**, phenylheptatriyne, was found in several hybrids from parents which did not contain it but which did have the closely related compound **5**, phenylhepta-diyne-ene.

In the three Type B crosses involving *B. cosmoides*, the F_1 produced the parental array of C_{13} acetylenes (compounds **1**, **3** and **5**) and three novel compounds - compound **4** , and compounds **8** and **10**, C_{17} hydrocarbons which are biosynthetically several steps removed from the C_{13} acetylenes (Table X). *Bidens molokaiensis* X *B. macrocarpa*

TABLE XII. POLYACETYLENE IN F_2 PLANTS

	Compounds					
Bidens	1	2	3	4	5	7
Cross 12 (B194)	+	-	-	-	+	-
B194s (F_2)	+	-	-	-	+	-
Cross 13 (B185)	+	-	-	-	+	-
B185s (F_2)	+	-	-	+	+	-

individuals synthesize C_{13} aromatic acetylenes **4** and **5** not found in *B. macrocarpa* which is characterized by C_{17} compounds **8** and **10** (Table X).

Type C crosses between two species of *Bidens* which produce leaf acetylenes result in F_1 individuals which express a combination of the major acetylenes in both parents (Table VIII). In two cases. *B. cervicata* X *B. cosmoides* and *B. torta* 17B X *B. hawaiensis* , compounds not found in either parent were expressed. In the cross *B. valida* X *B. macrocarpa,* the C_{17} acetylenes from *B. macrocarpa* were absent from the F_1 whereas there was additivity of parental arrays in *B. cervicata* X *B. macrocarpa* (Table XI).

F_2 individuals from the progeny of two Type B crosses (*B. sandvicensis* ssp. *confusa* X *B. mauiensis* - B185s, and *B. sandvicensis* ssp. *confusa* X *B. sandvicensis* ssp. *sandvicensis* - B194s) were analyzed for acetylenes. Every individual examined contained compounds **1** and **5**, the same compounds found in all the F_1 and in the leaves of *B. sandvicensis* ssp. *confusa* (Table XII).

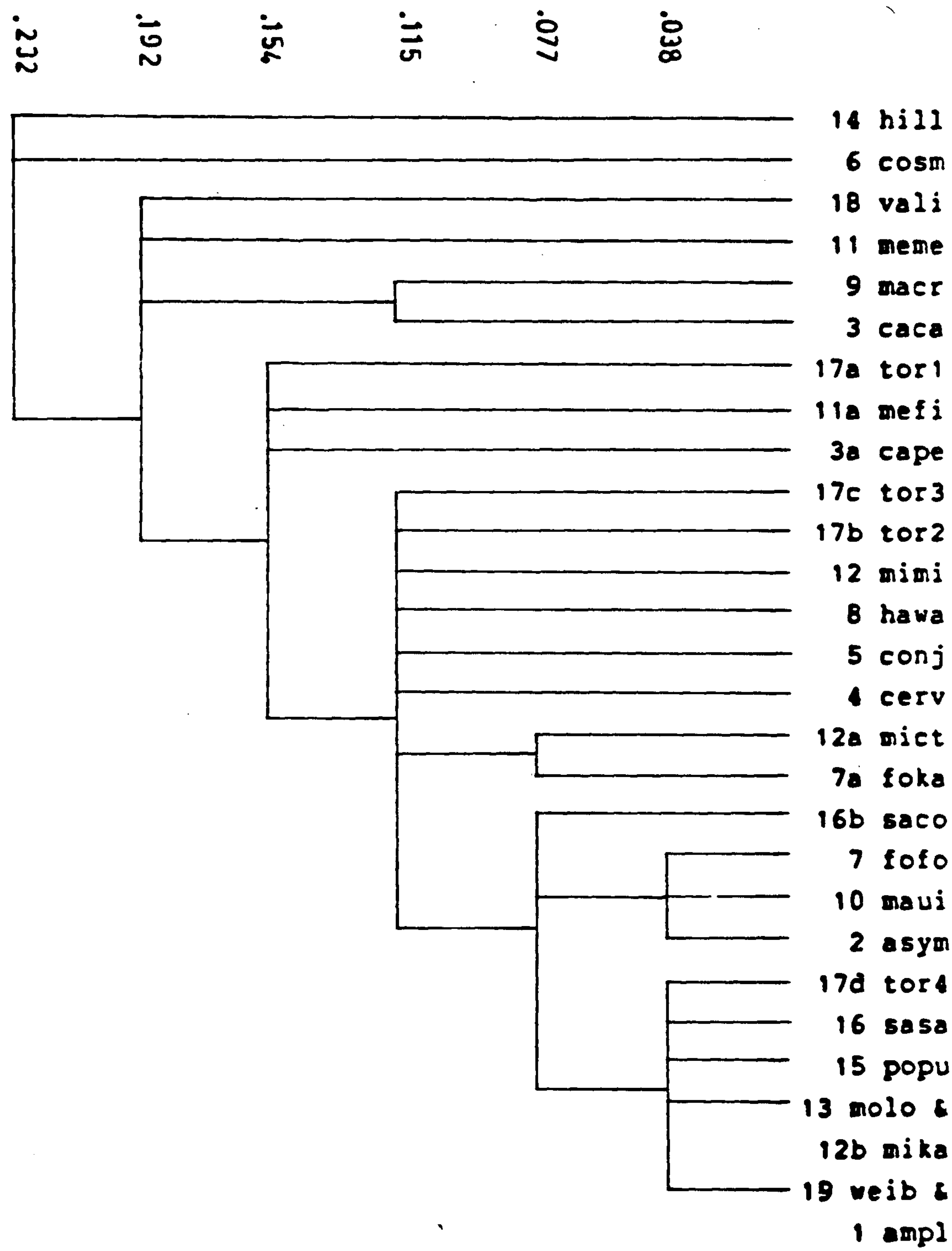
D. DISCUSSION

POLYACETYLENES IN *BIDENS* TAXA

As can be seen in Tables V and VI, there is no obvious overall pattern in the distribution of polyacetylenes in Hawaiian *Bidens*. A dendrogram of the taxa produced using the MIDAS statistical package with a simple matching similarity coefficient and a single linkage (nearest neighbour) clustering algorithm shows little hierarchical pattern above the species level (Figure 9). Many taxa cluster at the same level, and most levels added sequentially. Morphologically similar taxa do not often cluster together, e.g., *B.mauiensis* and *B.molokaiensis*, *B.cervicata* and *B.forbesii*, *B.conjuncta* and *B.micrantha* ssp. *micrantha*, or the subspecies of *B.menziesii*, *B.micrantha*, *B.campylotheca* and *B.sandvicensis*. The relative constancy of polyacetylenes within taxa and the absence of hierarchical structure in the classification above the species level provides little evidence of relationships among taxa but may be expected in a case of multiple divergences from a common ancestor.

Moreover, the relatively small array of compounds can be derived from a common unsaturated fatty acid precursor, oleic acid (Figure 7). Certainly this means that the Hawaiian *Bidens* share the enzyme system capable of dehydrogenating the olefinic bond, a capacity widespread in the Asteraceae, as well as the means to synthesize the aromatic acetylenic and thiophenic systems, already notable

FIGURE 9. DENDROGRAM OF TAXA BASED ON SIMILARITY OF POLYACETYLENES



in the Tribe Heliantheae (Heywood *et al.*, 1977).

A special feature which separates the Hawaiian group from its relatives is the consistent presence of a phenylthiophene derivative. This is significant in that it supports the biosystematic evidence indicating that all Hawaiian *Bidens* evolved from a single ancestral species. It would appear, however, that there has been less evolutionary diversification in polyacetylenes than in morphological characters although more differentiation than has occurred in isozymes.

All except four Hawaiian taxa can be distinguished by unique arrays of leaf and root acetylenes. *B.micrantha* ssp. *kalealaha* and *B.molokaiensis* both produce only 1 and 18; *B.amplectens* and *B.wiebkei* produce 1, 2 and 17 (Tables III, IV). These four taxa produce the smallest number of acetylenes of any of the *Bidens*, and since they are not very similar morphologically, their identical polyacetylene profiles probably do not reflect taxonomic relationships but are, rather, a result of the paucity of compounds.

Similarities in polyacetylenes among taxa do not correlate well with morphological similarities, and the recognition of related species groups on the basis of polyacetylenes does not appear to be possible. The various compounds seem to be randomly assorted among the taxa, as are many of the morphological differences noted by Gillet (1975). Distributions of particular acetylenes do show patterns of taxonomic significance, however. Qualitative variation in polyacetylenes was found within only one taxon. Although only one population of some taxa was available for analysis, two to five populations were analyzed for 16 of the taxa. All populations of *B.sandvicensis* ssp. *sandvicensis* have identical compounds even though this taxon as interpreted here includes seven species, two varieties and one forma according to Sherff (1937).

Bidens molokaiensis was treated as two species by Sherff, *B.molokaiensis* on Molokai (Figure 5), and *B.cuneata*, restricted to the top of Diamond Head on Oahu (Figure 3). They are decumbent, low spreading herbs whose supposed differences in the shape of their leaf bases and the number of teeth on the leaves disappeared when the plants were cultivated under the same conditions (Ganders and Nagata, 1983b) No acetylenes were found in the leaves and root acetylenes are identical.

Bidens mauiensis is very similar to *B.molokaiensis* except that it has winged achenes unique in Hawaiian *Bidens*, and highly variable leaf shape. Sherff recognized several varieties based on leaf shape differences, but they are now considered untenable (Ganders and Nagata, 1983b). All *B.mauiensis* specimens examined were uniform in the occurrence and distribution of polyacetylenes. *Bidens mauiensis* does differ consistently from *B.molokaiensis* by the presence of compounds **10**, **12** and **13** in its roots.

Variation within taxa was found only in *B.torta*, a morphologically variable taxon endemic to Oahu and widespread in distribution. Although its most distinctive feature is a twisted or coiled achene, plants can vary in the degree of coiling of achenes even within the same population. They can also vary in the amount and coloration of leaf pubescence and in the number of leaflets. Variants do not appear to have distinct geographical ranges. Individuals from four different populations of *B. torta* were found to possess different combinations of compounds. Populations 17A, 17B, and 17C are from the Waianae Range and 17D from the Koolau Range (Figure 3). Of these, 17A individuals appear to be unique among Hawaiian *Bidens* being the only plants in which phenylheptatriyne (PHT) is found. Their roots are also highly unusual in that no ene-tetrayne-ene (1) was detected, although **5** was present. Both 17B and 17D accumulate only **5** in the leaves; 17C did not have any aromatic acetylenes in the leaves, only the C_{17} hydrocarbons. The combinations of acetylenes in the roots were distinctive for all four populations. These differences remained consistent throughout the period of the study. Although *B. torta* is as variable in its polyacetylenes as it is in its morphology, the polyacetylenes appear to be characteristic of specific populations while morphological variation is not.

In contrast, no other taxon showed intra- or interpopulation differences. For example, *B. hawaiensis*, endemic to Hawaii, is a morphologically uniform and distinctive species although it occurs in ecologically variable sites. It may readily be recognized on the basis of leaves or flowers alone. All individuals from three populations examined were consistent in the pattern of polyacetylenes accumulated in the leaves and roots. The leaves contained only compound **18** and the roots produced all classes except the aromatic acetylenes.

In all cases examined the subspecies of a species differed in polyacetylene distribution. In some cases, subspecies which have rather subtle distinctions in morphological characters can easily be separated on the basis of polyacetylene differences.

Bidens sandvicensis is an extremely variable taxon which inhabits a wide range of habitats on Kauai and Oahu. Leaf shapes can be trifoliolate to bipinnately compound or divided into narrow ultimate segments, and all forms may be found in the same population. Many segregates recognized by Sherff (1937) on the basis of leaf forms are invalid according to Ganders and Nagata (1983b; 1984) and *B. sandvicensis* is presently considered to consist of ssp. *sandvicensis* and ssp. *confusa* (Ganders and Nagata, 1983b; 1984). These two taxa are often difficult to tell apart morphologically - subspecies *confusa* is a high elevation taxon restricted to the edge of Waimea Canyon in Kauai and tends to have larger ray flowers and flower heads, and narrower leaflets than ssp. *sandvicensis*. They may, however, be distinguished by the presence of phenyl-diyne-ene (5) in the leaves of ssp. *confusa* and the total absence of acetylenes in the leaves of ssp. *sandvicensis*. Five populations from Oahu and one population from Kauai of ssp. *sandvicensis*, and two populations of ssp. *confusa* were examined. The root acetylenes of the two subspecies are identical.

Bidens menziesii ssp. *menziesii* and ssp. *filiformis* are obviously more closely related to each other than to any other Hawaiian *Bidens* (Ganders and Nagata,1983b). They have characteristic leaves which vary in size but which are bipinnately divided into long linear segments less than 5mm wide. Both favour relatively arid, sunny and windswept areas, including the cinder cones and lava flows of Hawaii (Figures 4 to 6). The two subspecies are quite distinct morphologically however, and are allopatric on Maui and Molokai (ssp. *menziesii*) and Hawaii (ssp. *filiformis*). They are further distinguished by the absence of leaf acetylenes in ssp. *filiformis* and the presence of C_{17} compounds 8 and 9 in ssp. *menziesii*. There are no differences in root acetylenes.

Another example where leaf acetylenes separate two subspecies while root acetylenes are identical can be found in *B. campylotheca*. *Bidens campylotheca* consists of three subspecies, only two of which were examined in this study. Subspecies *campylotheca* and ssp. *pentamera* both had a similar range of acetylenes in the leaves except that ssp. *pentamera* also accumulates 5 and 7, aromatic acetylenes. The latter subspecies is restricted to the foggy rainforests above 1500m on East Maui (Figure 4) and differs morphologically from ssp. *campylotheca* primarily in leaf shape.

Bidens forbesii consists of two subspecies, ssp. *forbesii*, which occurs primarily along the north coast of Kauai, and ssp. *kahiliensis*, a montane wet forest form restricted to the vicinity of Mt. Kahili (Figure 2). Acetylenes are absent from the leaves of all six *B.forbesii* populations although two compounds, **5** and **12** , are found in the roots of ssp. *kahiliensis* but not in the roots of ssp. *forbesii*.

Most species of Hawaiian *Bidens* are separable on the basis of polyacetylene differences. For example, *B.forbesii* ssp. *kahiliensis* is sympatric with *B.valida* on Mt.Kahili, Kauai. They are very similar vegetatively although distinct in floral and achene characters. The two species are interfertile but they remain discrete taxa because they flower at different times of the year. While *B.forbesii* leaves lack polyacetylenes, those of *B.valida* contain several compounds, notably **5**, the 'phenyl-diyne-ene'. Plants grown from a mixed collection of cuttings from this locality were distinguished by analysis of leaf acetylenes and their identities later confirmed when the plants flowered. The roots of *B.valida* did not contain compound **5** and may be distinguished from those of *B.forbesii* ssp. *kahiliensis* on this basis.

Bidens cervicata is closely related to *B.forbesii* ssp. *forbesii*. Both taxa have identical achenes and prominently quadrangular stems, although *B.cervicata* has slightly larger flower heads, terminal inflorescences, and smaller, less succulent and more numerous leaflets which are narrower and more deeply serrate than those of *B.forbesii*. Some populations of *B.forbesii* ssp. *forbesii* from the north coast of Kauai (B12, B74, B101 and B124) have individuals which appear somewhat intermediate between *B.forbesii* and *B.cervicata* in morphology. The two species, however, can be separated on the basis of polyacetylene differences. *Bidens forbesii* does not accumulate acetylenes in the leaves but *B.cervicata* leaves contain compounds **5** and **7**. The roots of all *B.forbesii* populations sampled contain **12**, the C_{14}-tetrahydropyran, a compound not found in *B.cervicata*. Analysis of morphologically intermediate plants showed that they contained the acetylenes of typical *B.forbesii* ssp. *forbesii*, suggesting that the two species do not intergrade.

Other examples where morphologically similar species can be separated on the basis of polyacetylenes include *B.conjuncta* and *B.micrantha* ssp. *micrantha*. Both occur on West Maui and differ mainly in quantitative characters, but possess different polyacetylenes in their roots. *B.conjuncta* also produces compound **8** in its leaves while polyacetylenes are absent in the leaves of *B.micrantha*.

Morphologically, *B.asymmetrica* is a rather poorly defined taxon and is sometimes difficult to distinguish from

B.sandvicensis ssp. *sandvicensis* where their ranges are contiguous in the southern Koolau Range on Oahu. It is also rather similar to *B.torta* , which occurs in the northwestern Koolau Range. Although *B.torta* exhibits great interplant variation in polyacetylenes, these three species can be separated on the basis of their polyacetylenes.

Finally, *B.cosmoides* is a morphologically unique species endemic to Kauai. It has large flower heads with exserted styles which extend 20 - 25mm beyond the anthers, and achenes which are permanently enveloped by their subtending chaffy bracts, both unique features in the genus (Ganders and Nagata,1983a). It is sufficiently different from all other *Bidens* species that Sherff (1937) placed it in the monotypic section Degeneria. Gillett (1975) later proposed that there had been two separate introductions of *Bidens* to the Hawaiian Islands, one which gave rise to *B.cosmoides*, and the other to all the other *Bidens* species. This hypothesis was considered unlikely by Ganders and Nagata (1983b; 1984), because *B.cosmoides* can be crossed with all other Hawaiian *Bidens*. Therefore they most likely evolved from a single ancestor. The polyacetylenes of *B.cosmoides* indicate a close relationship with other Hawaiian taxa. Of the nine polyacetylenes found in leaves and roots of *B.cosmoides*, only compound **3** was not found in other Hawaiian taxa. Each of the other compounds was found in at least seven other taxa. The polyacetylene data supports a monophyletic origin for the Hawaiian species of

Bidens.

POLYACETYLENES IN *BIDENS* HYBRIDS

Few studies on the inheritance of polyacetylene production have been reported. Bistis and Anchel (1966) and Carey *et al*. (1974) examined the basidiomycete *Clitocybe truncicolor* which synthesizes trans-dehydromatricarianol, $CH_3-(C{\equiv}C)_3CH$ $CHCH_2OH$, and its methyl ether. Individual homokaryons exhibited definite and reproducible differences in polyacetylene production. These differences were evident in the progeny of crosses between distinctive homokaryons and the levels of polyacetylenes produced were correlated with specific mating types. The study in 1966 was the first to provide experimental evidence for genetic control of polyacetylene synthesis.

Norton (1984) performed a similar investigation using *Bidens alba* L. var. *radiata* (Schz. Bip.) Ballard and *B. pilosa* var. *minor* (Blume) Sherff. *Bidens alba* synthesizes phenylheptatriyne (PHT or compound **4** in this paper) in its leaves whereas acetylenes are absent from *B. pilosa* leaves. PHT was found in the leaves of all F_1 individuals resulting from *B. alba* X *B. pilosa* but at levels which were less than half of that in *B. alba*. PHT synthesis segregated in the F_2 generation although the ratios of segregants did not agree with expected values and individual values were much lower than anticipated if PHT levels are a function of gene dosage.

In the present study, the inheritance of polyacetylene biosynthesis in Hawaiian *Bidens* was examined. All Hawaiian *Bidens* produce acetylenes in their roots, but only 15 taxa express this ability in the leaves. Biosynthesis in leaves and roots appear to be independent (Van Fleet, 1970) and occurs *de novo* in the leaves (see Chapter III). Most of the Hawaiian species can be separated on the basis of their leaf acetylene arrays and selected hybrids were used for this analysis. Quantitative levels of acetylenes produced were not measured.

The only other study of this nature was reported by Van Fleet(1970). He worked with *Coreopsis* and examined the genetics of polyacetylene formation by the endodermis of roots, stems and leaves of *C. saxicola* Alexander, *C. grandiflora* Hogg ex. Sweet and their artificial and natural hybrids. The stems and leaves of the two parent species produced mainly PHT but in some forms of *C. grandiflora* and in most of the artificial hybrids, a mixture of the trideca-triene-triyne (compounds **11** and **12**) and the phenylhepta-diyne-ene (compounds **5** and **6**) was produced. An entity Van Fleet calls a 'general ecotype', which is interpreted here as natural hybrids in general, contains mixtures of compounds **4**, **5** and **6** in its stems and leaves. The roots of *C. saxicola* and *C. grandiflora* produce predominantly the trideca-ene-tetrayne-ene (compounds **1** and **2**) and "....compounds produced in the roots of the hybrids are predominantly the same as the parents."

Biosynthesis in the aerial tissues seems to be more variable than in the roots. *Coreopsis* 'hybrid ecotypes' could not be distinguished or separated on the basis of acetylenes produced in the roots but could be distinguished from parental types on the basis of leaf acetylenes. The validity of Van Fleet's data may be questioned because the information provided is descriptive and statistically nonspecific. Nevertheless, it does indicate that the genetics of polyacetylene biosynthesis in higher plants is a challenging problem.

Data from a preliminary investigation of Hawaiian *Bidens* hybrids suggests that acetylene biosynthesis *per se* in leaves is a heritable and dominant phenotype. Polyacetylenes were found in the leaves of progeny from Type B and Type C crosses but not from Type A crosses (Tables VII to XI). Acetylene synthesis was not segregated in the small number of F_2 individuals examined from Type B crosses. Instead, all plants produced the acetylenes found in *B. sandvicensis* ssp. *confusa* (Table XII). Whether there was significant variation in the quantitative levels of compounds produced is not known.

In general, Hawaiian *Bidens* produce a limited array of acetylenes, consisting of C_{17} and C_{13} compounds. According to the scheme proposed here, these compounds may be commonly derived from oleic acid but are subsequently elaborated along biosynthetically divergent pathways (Figure II-7). In the leaves, most species tend to produce predominantly C_{13} or C_{17} compounds. With the exception of crosses involving *B. cosmoides* and *B. macrocarpa*, Type B progeny produced only compounds in the parental class. In *B. cosmoides* hybrids, C_{17} compounds not found in the parents were synthesized, and in *B. macrocarpa* hybrids, C_{13} aromatic compounds absent from the parental array were observed. Data from Type C crosses is similar. There was additivity of parental polyacetylenes in F_1 progeny but there was also synthesis of C_{17} compounds when only C_{13} arrays were expected and *vice versa*. This is not surprising since one would expect that the complete set of instructions for *de novo* acetylene synthesis exists in the leaf genome. Moreover, regulation and control of genetic expression is further complicated by the polyploid condition of Hawaiian *Bidens* (2N=72; X=12) (Mears, 1980; Fedorov, 1974; Gillett and Lim, 1970; Skottsberg, 1953).

That only certain compounds are expressed in significant amounts in each species could be due to any number of factors. Certain enzymes along the sequence may have depressed activity or may be absent, the pool size of key precursors and intermediates, and the turnover rates of the end products would affect the direction of equilibrium in the synthetic sequence. In addition, the sequence does not exist in isolation and the level of its activity would be influenced by the state of primary processes such as fatty acid metabolism. Whatever the governing factors, it is clear that the *status quo* is altered when *Bidens* are hybridized.

E. CONCLUSION

The leaves and roots of Hawaiian species of *Bidens* accumulate a moderate diversity of polyacetylenes which may all be biosynthetically related. Of these compounds, the phenylthiophenes **14** to **17** appear to be ubiquitous and unique to the species. This is consistent with other evidence that the Hawaiian species are all derived from a single ancestral immigrant to the Hawaiian Islands. There has been less evolutionary diversification in polyacetylenes than in morphology and ecology in Hawaiian *Bidens*, but greater differentiation than is found in isozymes.

Polyacetylenes are usually constant within a given taxon. Only *B. torta* exhibited interpopulational variation in compounds accumulated. Nearly all taxa can be distinguished by the array of acetylenes in roots and leaves although species specific compounds are rare. Even subspecies which are difficult to distinguish morphologically can be unequivocally identified on the basis of their polyacetylenes. The distribution of polyacetylenes in the populations studied strongly supports the species concepts of Ganders and Nagata (1983b,1984) based on morphological and ecogeographical data.

Subspecies of the same species exhibited as many differences in polyacetylenes as did different species. Above the level of subspecies and species, polyacetylenes were not correlated with relationships based on morphology. Therefore, it is not yet possible to define species groups within Hawaiian *Bidens* based on correlated morphological and chemical characters. Adaptive radiation in Hawaiian *Bidens* has produced a group of species that combine an assortment of morphological and chemical characters which occur in a large number of combinations.

The *de novo* synthesis of polyacetylenes in *Bidens* leaves is a heritable and dominant trait. Acetylenes were expressed in the leaves of hybrids with at least one leaf acetylene-producing parent. Synthesis, however, was not segregated in the small number of F_2 individuals examined, all of which produced the parental arrays.

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III. BIOSYNTHESIS OF POLYACETYLENES FROM $^{14}CO_2$

A. INTRODUCTION

Natural polyacetylenes comprise a wide range of combinations of differing chain lengths (C_6 - C_{18}), degrees of unsaturation, and a considerable number of functional groups and cyclic systems in varying relationship to the chromophores (Jones and Thaller, 1978).

The almost exclusive occurrence of straight carbon chains from C_{18} down suggests that the biosynthesis of polyacetylenes is a variant of that of fatty acid synthesis from acetate, and there is abundant experimental evidence to support this assumption (e.g.,Bu'Lock and Gregory, 1959; Bu'Lock *et al.*, 1961; Bu'Lock and Smith, 1962; 1963; Jones , 1966; Bohlmann and Jente, 1966; Fairbrother *et al.*, 1967). The currently accepted hypothesis for the biogenesis of polyacetylenes in plants was first proposed by Bu'Lock (1966). This primarily involves the desaturation of the distal half (C_{10} - C_{18}) of oleic acid via the α-en-δ-yne system of crepenynic acid.

Subsequent transformations include chain-shortening (usually by the classical α- or β-oxidations of fatty acids at the carboxyl end), rearrangement and/or oxidation of the conjugated system, extension of the chromophore, chain-shortening at the distal end by deformylation or decarboxylation, functionalization and cyclization (Jones and Thaller, 1978). The exact sequence of reactions would be characteristic of the organism and its physiology, producing a variety of acetylenes depending on the type and availability of enzymatic substrates.

Hawaiian *Bidens* species synthesize a limited array of C_{13} and C_{17} polyacetylenes in their leaves and roots (Tables III, IV, V, VI). All these compounds may be theoretically derived from oleic acid in the sequence of reactions outlined in Figure 7. The presence of non-parental acetylenes in the leaves of F_1 progeny from *Bidens* crosses may be explained using this biogenetic scheme (Chapter II), thus demonstrating its utility. Although only half the taxa of Hawaiian *Bidens* synthesize leaf acetylenes, this ability has been shown here as a dominant and heritable phenotype. Since all the native species are believed to have evolved from a common ancestor (Ganders and Nagata, 1983a; 1983b; 1984; Helenurm and Ganders, 1985; Marchant *et al*., 1984), all presumably had the genetic information for acetylene biosynthesis which some taxa do not express in the leaves.

One of the objectives of this study was to establish that *de novo* polyacetylene synthesis occurs in the leaves of *Bidens* independently of the system in the roots. This was followed by investigation of the kinetics of acetylene accumulation in intact plants with comparisons of species producing different leaf acetylenes and an assessment of the relative efficiency of acetylene synthesis from $^{14}CO_2$. This would provide some indication of the practicality of synthesizing ^{14}C-labelled acetylenes using whole plants.

Finally, the accumulation and distribution of polyacetylenes in *Bidens* seedlings was determined using *B. alba* as a representative species.

B. MATERIALS AND METHODS

BIOSYNTHESIS OF POLYACETYLENES FROM $^{14}CO_2$ IN *BIDENS*

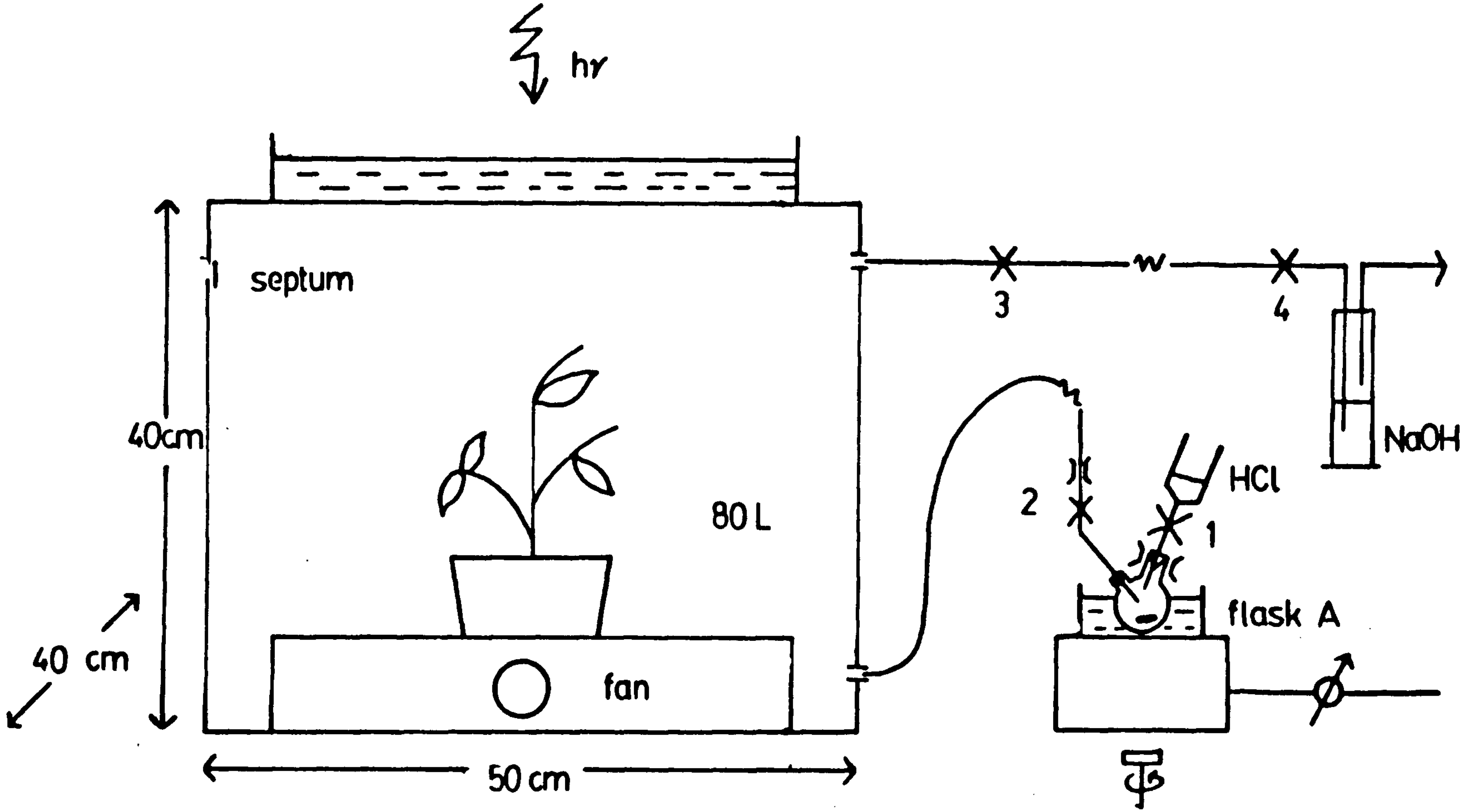
PLANT MATERIAL

Bidens cosmoides, *B. hillebrandiana* ssp. *polycephala*, *B. molokaiensis* and *B. alba* var. *radiata* were grown in UBC greenhouses under standard conditions. With the exception of *B. molokaiensis*, plants used for $^{14}CO_2$ feeding experiments were approximately six months old.

ADMINISTRATION OF $^{14}CO_2$

Whole, freshly watered plants were placed inside the photosynthetic chamber illustrated in Figure 10. The pots and soil surfaces were first covered with aluminum foil. Plants were allowed to equilibrate in the closed chamber for 30 minutes to one hour before each experiment. The entire chamber was set up in a fume hood with the overhead fluorescent lights switched on. A tungsten lamp emitting white light with an intensity of 303.57 ± 48 joules sec^{-1} cm^{-2} in the chamber was the major source of radiation. A tray of water, 50 cm in depth, was kept between the light source and the plants in order to minimize temperature fluctuations within the box. A small fan circulated the air within the chamber and temperature during $^{14}CO_2$ administration remained constant at 23°C.

FIGURE 10. $^{14}CO_2$ - FEEDING APPARATUS



All valves were closed at the beginning of each experiment and the chamber then partially evacuated through stopcock 3 for 1.5 minutes. Stopcock 3 was then closed for the rest of the feeding. Three hundred microlitres or 500μL of aqueous $NaH^{14}CO_3$ (53.0 μCi/umole, New England Nuclear) was pipetted into flask A equipped with a small magnetic stir bar. Equimolar quantities of concentrated HCl were added to the $NaH^{14}CO_3$ solution and generation of $^{14}CO_2$ according to the equation:

$$NaH^{14}CO_3 + HCl(conc) \longrightarrow H_2O + {}^{14}CO_2 + NaCl$$

allowed to proceed. The reaction flask was kept in a warm water bath (35-40°C). Stopcocks 1 and 2 were opened and $^{14}CO_2$ flushed into the photosynthetic chamber by allowing atmospheric air in via the $^{14}CO_2$-generating apparatus. When chamber pressure was equalized with atmospheric pressure, stopcock 1 was closed.

Plants were allowed to metabolize for one hour in the $^{14}CO_2$-enriched environment, at the end of which the $^{14}CO_2$ generator was disconnected and the unused $^{14}CO_2$ evacuated through stopcock 3 into 1.0M NaOH, where it was trapped as $NaH^{14}CO_3$. The rate of $^{14}CO_2$ utilization during each feeding period was measured by taking three 0.2 mL samples of chamber air through a rubber septum at 15 minute intervals. $^{14}CO_2$ was dissolved in Oxifluor-CO_2 (New England Nuclear) and subsequently counted for radioactivity.

MEASUREMENT OF ^{14}C UPTAKE INTO POLYACETYLENES

In all experiments a 60-minute pulse of $^{14}CO_2$ was given to plants in the chamber. Leaf samples were then taken from the plants immediately after $^{14}CO_2$ administration and at predetermined time intervals thereafter. Plants were allowed to photosynthesize in atmospheric air for 12, 24 and 168 hours after the radioactive pulse. Roots were sampled periodically as plants became available. All samples were extracted for polyacetylenes according to the method described in Chapter II.

Aliquots of MeOH and PE fractions were counted for radioactivity in 10 mL of Aquasol 2 (New England Nuclear). The light petroleum (PE) extracts were fractionated on preparative TLC plates (Merck, SG 60 F-254 0.25mm, 0.5mm and 2.0mm thick, 20 X 20cm) and respective polyacetylenes eluted off the silica gel with PE (30-60°C). All samples were run with purified acetylenes as reference compounds. The concentrations (C) of polyacetylenes were calculated from the absorbance (A) at the wavelength of maximum absorbance and the molar extinction coefficient of the compounds according to the formula

$$\epsilon = A\lambda/C.l$$

where 'l' is the length of the sample cell and equals 1cm (Parikh, 1974).

Samples were dried and resuspended in 10mL of Aquasol 2 and placed in a PDS/3-ISOCAP/300 (Searle) liquid scintillation counter. Radioactivity, measured as counts per

FIGURE 11. ^{14}C - EFFICIENCY CURVE

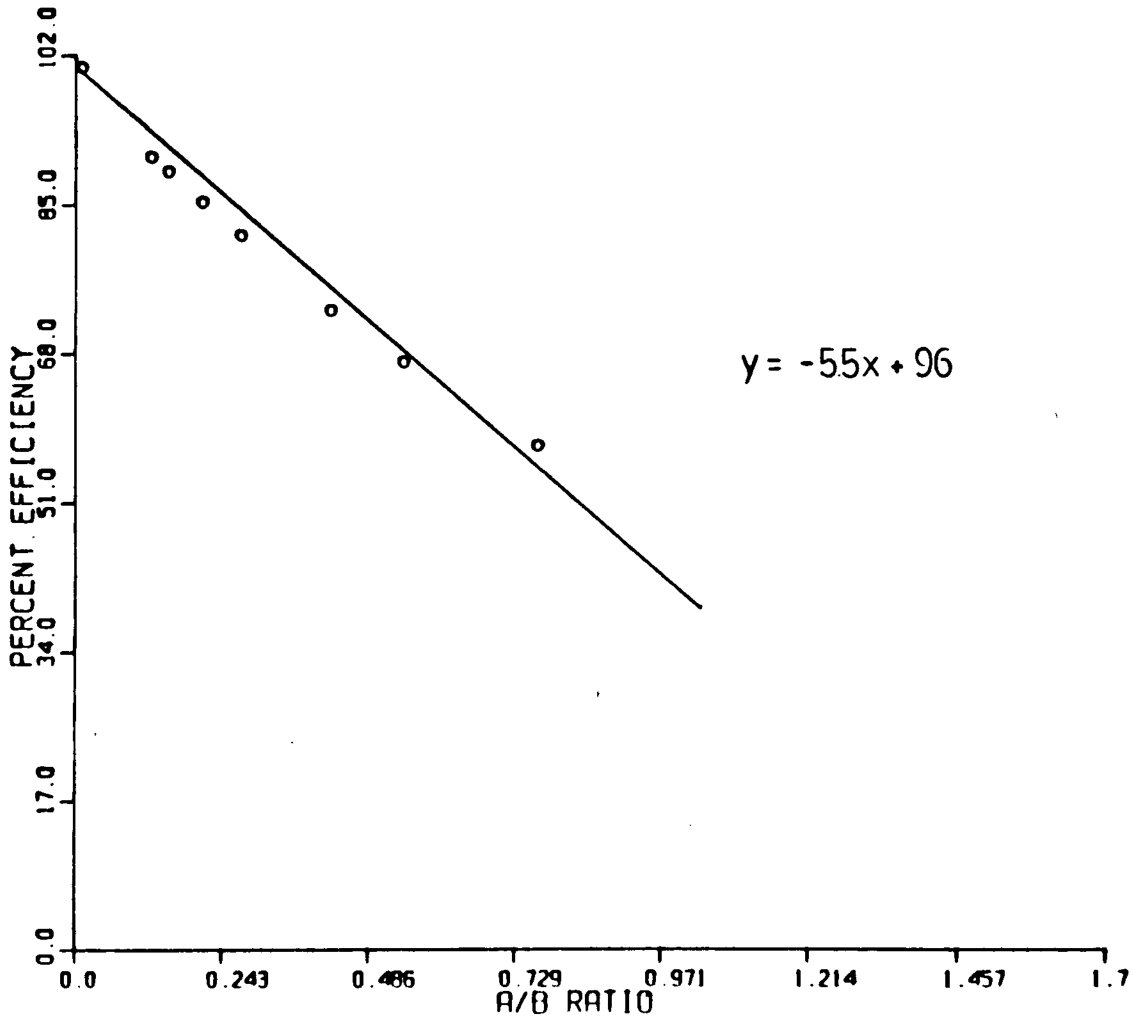


TABLE XIII. PERCENT QUENCHING OF RADIOACTIVITY BY POLYACETYLENES 1, 4 AND 5.

Compound ug	Percent decrease in dpm. 4	5	1
0	0.16	0.00	0.17
10	0.46	0.38	0.00
50	0.00	0.22	0.00
100	0.29	0.16	0.05
300	0.00	0.23	0.00
500	0.00	0.00	0.00
800	0.00	0.02	0.07
1000	4.54	0.00	0.36

minute (cpm) and the disintegrations per minute (dpm) of each sample, corrected for background, was calculated from a standard ^{14}C-efficiency curve (Figure 11). Quenching effects of the three acetylenes examined here (**1**,**4** and **5**) were also prepared and are shown in Table XIII.

KINETIC STUDIES

The uptake of ^{14}C into the methanol and light petroleum fractions and polyacetylenes in leaves of the four *Bidens* species was determined for 12 hours and 168 hours (one week) after $^{14}CO_2$ administration. In the 12 hour studies, samples were taken at two hour intervals and in the week long studies, samples taken once a day. Specific activity for the MeOH and PE fractions was expressed per unit fresh weight and for acetylenes as dpm/mg compound. Total amounts of acetylenes extracted were calculated for each time period and values expressed per gram of leaves. *Bidens alba* was used in 24 hour time course studies where samples were taken at two hour intervals.

STATISTICAL ANALYSIS

Raw data from the 24 hour time course tracer experiments using *B.alba* were transformed and subjected to analysis of variance using the programme UBC Anovar (1978). In each experiment, 3 plants and 3 replicates of MeOH and PE fractions per plant were available for 13 time intervals. Samples for each plant subsequently had to be combined for isolation of PHT because of the low level of radioactivity present. Acetylene calculations are based on readings from all plants at each time interval sampled. Data analysis was thus complicated by uneven cell sizes and two separate statistical comparisons were made.

Run 1: Three-level nested anova design for ^{14}C uptake into MeOH and PE fractions.

Run 2: Two-level nested anova design for ^{14}C uptake into PHT.

Four species of *Bidens*, *B. hillebrandiana*, *B. cosmoides*, *B. alba* and *B. molokaiensis* were used in the 12 hour and 168 hour studies. Only one plant was available per experiment and 3 replicates for MeOH and PE fractions had to be combined into one aliquot for polyacetylene purification. Subsequently, only one set of data was available for acetylenes at each time sampled and was not analyzed for variance.

ACCUMULATION AND DISTRIBUTION OF PHENYLHEPTATRIYNE IN *BIDENS ALBA* SEEDLINGS

Seeds of *B. alba* collected from greenhouse plants were germinated on damp filter paper in covered sterile petri dishes at 22°C. Seedlings were kept in the dark for one week and then allowed to develop under white light (15 hour photoperiod/day, "Universal White" fluorescent tubes). Whole seedlings were harvested at days 2, 4 and 5 of germination and leaves, hypocotyls and roots at days 7, 15, 20 and 24. All samples were weighed and crude light petroleum (BP 30-60°C) fractions prepared according to the method described in Chapter II. Dried PE fractions were resuspended in 1.0mL spectral grade MeOH and 20uL samples fractionated by high performance liquid chromatography (HPLC) on a Varian MCH-10 reverse phase column and Varian Model 5000 HPLC with Varian Series 634 variable wavelength UV detector.

The UV detector was set at 250nm which is the wavelength of maximum absorption (ϵ = 167,000) for phenylheptatriyne (PHT). Separation of the crude fractions was achieved at a flow rate of 1.0mL/min with a solvent gradient proceeding from 70% CH_3CN/20% H_2O to 100% CH_3CN in 15 minutes. Retention time (R_T for PHT under these conditions was 11.94 mins. ($\simeq$ 93% CH_3CN). Quantitation of eluted peaks was performed by an SP4100 Integrator (Spectra-Physics) and these values expressed per unit fresh weight of tissue.

C. RESULTS

BIOSYNTHESIS OF POLYACETYLENES FROM $^{14}CO_2$ IN *BIDENS* LEAVES

Three species of Hawaiian *Bidens*, *B. cosmoides*, *B. hillebrandiana* ssp. *polycephala*, *B. molokaiensis* and *B. alba* var. *radiata* were used in a series of time course tracer studies of polyacetylene biosynthesis. Choice of Hawaiian species was limited by the availability of healthy young plants which produce easily detectable leaf acetylenes in acceptable quantities. *Bidens cosmoides* leaves contain measurable amounts of compounds 1 and 5 (Table V) and were used in order to assess the partitioning of ^{14}C into these acetylenes. *Bidens hillebrandiana* which produces mainly compound 1 (Table V), and *B. molokaiensis*, which does not have leaf acetylenes, were used as comparisons. *Bidens alba* produces PHT (4) in its leaves and numerous plants were grown from seeds in UBC greenhouses. Experiments with Hawaiian species were repeated twice and those with *B. alba* four times.

In all experiments, plants were allowed to photosynthesize for 60 minutes in a $^{14}CO_2$-enriched atmosphere within the enclosed chamber (Figure 10). $^{14}CO_2$ disappearance during the 60 minute pulse was monitored and the data presented in Table XIV. Conversion of $NaH^{14}CO_3$ to $^{14}CO_2$ was nearly complete (99.42%) for all experiments and total uptake of ^{14}C into the plants and percent ^{14}C incorporation into polyacetylenes shown in Table XV.

TABLE XIV. $^{14}CO_2$ UPTAKE DURING 60 MINUTE PULSE-LABELLING OF *B. ALBA**

Time (mins)	Total $^{14}CO_2$ in Chamber (dpm)
0	$5.44 \times 10^6 \pm 6.2\%$
15	$4.54 \times 10^6 \pm 3.5\%$
30	$2.80 \times 10^6 \pm 7.2\%$
45	$2.12 \times 10^6 \pm 2.2\%$
60	$1.32 \times 10^6 \pm 3.8\%$

0.20mL air/sample (0.032% CO_2)

or 0.0064 mL CO_2/sample

total volume $^{14}CO_2$ in chamber (80L) = 25.6mL

therefore: total $^{14}CO_2$ in chamber at sampling =

dpm/0.0064mL X 25.6mL

* average of 4 experiments

TABLE XV. EFFICIENCY OF ^{14}C UPTAKE*

Percent conversion of $NaH^{14}CO_3$ to $^{14}CO_2$	99.4%
Total $^{14}CO_2$ in chamber:	
from 300 uCi $NaHCO_3$	6.73 X 10^8 dpm (298 uCi)
from 500 uCi $NaHCO_3$	11.28 X 10^8 dpm (499 uCi)
Percent total ^{14}C uptake	1.0% (0.1% - 2.4%)
Percent incorporation of ^{14}C into acetylenes	2.9% (0.1% - 12.9%)

* data from 15 experiments

The uptake of ^{14}C into the MeOH and PE fractions and the polyacetylenes in leaves of the four *Bidens* species was determined for 12 hours and 168 hours (one week) after $^{14}CO_2$ administration. In the 12 hour studies, samples were taken at two hour intervals and in the week long studies, samples taken once a day. Total amounts of acetylenes extracted were calculated for each time period and values expressed per gram fresh weight of leaves. These results are shown in Tables XVI to XXIII and Figures 12 to 21. In general, 0.1 to 2.0 percent of the administered radioactivity was incorporated into the methanol fractions and 0.02 to 12.9 percent of this recovered in the polyacetylenes.

Bidens alba was used in 24 hour time course studies where samples were taken at two hour intervals. Uptake of ^{14}C into *B. alba* leaves is shown in Table XXIV. Less than 0.1 percent of the administered label was incorporated into the methanol fraction, from which 0.22 percent went into PHT. Analysis of variance for this data is reported in Tables XXV and XXVI. The 24 hour accumulation of ^{14}C-PHT in *B. alba* leaves was statistically significant. Differences between samples within each time period for both MeOH and PE components were also significant. This is clearly due to technical problems inherent in the extraction procedure.

All the *Bidens* species used in these studies produce the ene-tetrayne-ene (1) in the roots. *De novo* acetylene biosynthesis in roots and leaves seem to occur independently (Van Fleet, 1970) but this does not preclude translocation

TABLE XVI. TWELVE HOUR UPTAKE OF ^{14}C INTO PHT BY *B. ALBA* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	PHT dpm/mg	PHT ug/gFW
1	$3.38 \times 10^{6} \pm 5.7\%$	$15.26 \times 10^{3} \pm 1.9\%$	$400 \pm 2.4\%$	308
3	$3.32 \times 10^{5} \pm 6.5\%$	$9.79 \times 10^{1} \pm 3.0\%$	$2600 \pm 1.9\%$	230
5	$1.16 \times 10^{5} \pm 2.4\%$	$3.96 \times 10^{1} \pm 8.9\%$	$600 \pm 8.1\%$	298
7	$1.31 \times 10^{6} \pm 4.4\%$	$26.81 \times 10^{3} \pm 6.5\%$	$6000 \pm 2.5\%$	237
9	$1.17 \times 10^{6} \pm 5.1\%$	$5.68 \times 10^{3} \pm 8.4\%$	$38700 \pm 6.3\%$	266
11	$0.77 \times 10^{6} \pm 3.2\%$	$5.54 \times 10^{3} \pm 7.4\%$	$4100 \pm 2.9\%$	156
13	$0.75 \times 10^{6} \pm 6.2\%$	$30.77 \times 10^{3} \pm 6.0\%$	$62500 \pm 4.5\%$	189

Total percent ^{14}C incorporation into MeOH fraction = 2.02

Total percent ^{14}C incorporation into PHT fraction =0.38

TABLE XVII. TWELVE HOUR UPTAKE OF ^{14}C INTO ENE-TETRAYNE-ENE (1) OF *B. HILLEBRANDIANA* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	1 dpm/mg	1 ug/gFW
1	$5.32 \times 10^5 \pm 6.1\%$	$424 \pm 8.3\%$	$600 \pm 2.1\%$	5.12
3	$3.84 \times 10^6 \pm 3.2\%$	$227 \pm 4.4\%$	$1400 \pm 2.5\%$	3.31
5	$1.61 \times 10^6 \pm 3.4\%$	$197 \pm 4.5\%$	$4000 \pm 4.8\%$	1.62
7	$0.92 \times 10^5 \pm 2.1\%$	$309 \pm 5.2\%$	$52500 \pm 5.2\%$	1.08
9	$2.28 \times 10^5 \pm 1.9\%$	$87 \pm 3.1\%$	$9200 \pm 3.1\%$	0.95
11	$2.26 \times 10^5 \pm 3.0\%$	$229 \pm 6.9\%$	$12600 \pm 3.2\%$	0.93
13	$1.19 \times 10^5 \pm 3.4\%$	$197 \pm 4.2\%$	$6400 \pm 5.5\%$	1.91

Total percent ^{14}C incorporation into MeOH fraction = 0.45%

Total percent ^{14}C incorporation into Compound 1 = 0.023%

TABLE XVIII. TWELVE HOUR UPTAKE OF ^{14}C INTO ACETYLENES **1** AND **5** OF *B. COSMOIDES* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	1 dpm/mg	5 dpm/mg	total acetylenes ug/gFW
1	$4.89 \times 10^{5} \pm 3.5\%$	$27 \pm 1.5\%$	$100 \pm 4.8\%$	$600 \pm 4.8\%$	102
3	$9.16 \times 10^{6} \pm 6.1\%$	$565 \pm 3.4\%$	$600 \pm 3.1\%$	$1600 \pm 6.7\%$	33
5	$4.65 \times 10^{5} \pm 2.7\%$	$675 \pm 2.4\%$	$400 \pm 2.9\%$	$800 \pm 2.8\%$	128
7	$5.41 \times 10^{6} \pm 3.2\%$	$1946 \pm 2.2\%$	$1900 \pm 6.9\%$	$2100 \pm 5.2\%$	28
9	$2.84 \times 10^{5} \pm 3.11\%$	$3221 \pm 1.3\%$	$5600 \pm 7.2\%$	$6200 \pm 5.9\%$	19
11	$1.04 \times 10^{6} \pm 5.2\%$	$1936 \pm 6.7\%$	$1800 \pm 7.1\%$	$2600 \pm 6.5\%$	47
13	$0.91 \times 10^{5} \pm 4.8\%$	$2153 \pm 8.7\%$	$4700 \pm 5.8\%$	$9000 \pm 4.5\%$	20

Total percent ^{14}C incorporation into MeOH fraction = 1.01

Total percent ^{14}C incorporation into Compound **1** = 0.089

Total percent ^{14}C incorporation into Compound **5** =0.044

Total percent ^{14}C incorporation into total acetylenes = 0.13

TABLE XIX. TWELVE HOUR UPTAKE OF ^{14}C INTO MEOH AND PE FRACTIONS OF *B. MOLOKAIENSES* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW
1	$4.64 \times 10^5 \pm 5.9\%$	$496 \pm 2.6\%$
3	$2.73 \times 10^5 \pm 2.4\%$	$933 \pm 4.8\%$
5	$3.81 \times 10^5 \pm 5.8\%$	$240 \pm 9.2\%$
7	$1.06 \times 10^5 \pm 4.1\%$	$1115 \pm 5.8\%$
9	$1.79 \times 10^5 \pm 5.9\%$	$393 \pm 9.7\%$
11	$3.27 \times 10^5 \pm 3.1\%$	$2633 \pm 8.9\%$
13	$2.09 \times 10^5 \pm 4.4\%$	$3090 \pm 6.3\%$

Total percent ^{14}C incorporation into MeOH fraction= 0.41%

TABLE XX. ONE WEEK UPTAKE OF ^{14}C INTO PHT BY *B. ALBA* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	PHT dpm/mg	PHT ug/gFW
25	$1.43 \times 10^{9} \pm 2.6\%$	$3618 \pm 4.5\%$	$4700 \pm 2.5\%$	59
49	$2.51 \times 10^{9} \pm 2.3\%$	$3486 \pm 7.5\%$	$12100 \pm 3.8\%$	42
73	$3.97 \times 10^{9} \pm 7.6\%$	$12920 \pm 7.5\%$	$23800 \pm 3.4\%$	157
97	$2.57 \times 10^{9} \pm 4.7\%$	$7311 \pm 1.4\%$	$17700 \pm 5.9\%$	70
169	$0.36 \times 10^{9} \pm 2.4\%$	$111 \pm 3.2\%$	$20300 \pm 6.2\%$	109

Total percent ^{14}C incorporation into MeOH fraction =0.15

Total percent ^{14}C incorporation into PHT = 12.8

TABLE XXI. ONE WEEK UPTAKE OF ^{14}C INTO ENE-TETRAYNE-ENE (1) OF *B. HILLEBRANDIANA* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	1 dpm/mg	1 ug/gFW
25	1.28×10^{6} ± 4.1%	464 ± 6.9%	41300 ± 6.2%	0.37
49	1.58×10^{6} ± 4.5%	2595 ± 8.5%	92100 ± 3.5%	0.80
73	0.89×10^{6} ± 3.9%	1105 ± 5.3%	40600 ± 9.2%	0.97
145	2.49×10^{5} ± 9.8%	128 ± 7.0%	25800 ± 6.2%	0.94
169	0.79×10^{5} ± 3.5%	794 ± 7.3%	46100 ± 3.1%	0.38

Total percent ^{14}C incorporation into MeOH fraction = 0.17

Total percent ^{14}C incorporation into Compound 1 = 0.20

TABLE XXII. ONE WEEK UPTAKE OF ^{14}C INTO ACETYLENES **1** AND **5** OF *B. COSMOIDES* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	**1** dpm/mg	**5** dpm/mg	Total ug/gFW
25	$4.59 \times 10^5 \pm 4.9\%$	$2.25 \times 10^4 \pm 4.4\%$	$1.6 \times 10^4 \pm 2.3\%$	$2.6 \times 10^4 \pm 1.8\%$	35
49	$6.66 \times 10^5 \pm 8.0\%$	$1.26 \times 10^4 \pm 6.8\%$	$6.7 \times 10^4 \pm 5.2\%$	$4.52 \times 10^4 \pm 3.9\%$	9
73	$3.10 \times 10^5 \pm 5.2\%$	$0.91 \times 10^4 \pm 6.8\%$	$3.54 \times 10^4 \pm 8.1\%$	$2.88 \times 10^4 \pm 4.5\%$	13
145	$0.42 \times 10^6 \pm 1.8\%$	$0.18 \times 10^4 \pm 1.1\%$	$3.15 \times 10^4 \pm 6.6\%$	$0.49 \times 10^4 \pm 4.8\%$	47
169	$3.10 \times 10^5 \pm 9.2\%$	$1.41 \times 10^4 \pm 2.4\%$	$3.48 \times 10^4 \pm 6.1\%$	$0.72 \times 10^4 \pm 6.2\%$	24

Total percent ^{14}C incorporation into MeOH fraction = 0.55

Total percent ^{14}C incorporation into Compound **1** = 0.59

Total percent ^{14}C incorporation into Compound **5** = 0.33

Total percent ^{14}C incorporation into total acetylenes = 0.93

TABLE XXIII. ONE WEEK UPTAKE OF ^{14}C INTO MEOH AND PE FRACTIONS OF *B. MOLOKAIENSIS* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW
25	$1.15 \times 10^5 \pm 4.4\%$	$1042 \pm 6.2\%$
49	$1.49 \times 10^5 \pm 8.5\%$	$547 \pm 7.5\%$
73	$0.51 \times 10^5 \pm 9.1\%$	$324 \pm 1.9\%$
97	$7.38 \times 10^5 \pm 9.0\%$	$907 \pm 5.4\%$
169	$0.86 \times 10^5 \pm 2.8\%$	$8686 \pm 1.0\%$

Total Percent ^{14}C incorporation into MeOH fraction = 0.07%

TABLE XXIV. TWENTY-FOUR HOUR UPTAKE OF ^{14}C INTO PHT BY *B. ALBA* LEAVES

Time (hours)	MeOH dpm/gFW	PE dpm/gFW	PHT dpm/mg	PHT ug/gFW
1	2.94 X 10^5 ± 7.8%	1224 ± 5.2%	11.23 ± 6.4%	373
3	1.91 X 10^5 ± 2.8%	614 ± 3.7%	10.2 ± 5.0%	325
5	1.95 X 10^5 ± 8.3%	1271 ± 8.5%	19.2 ± 3.2%	455
7	2.48 X 10^5 ± 6.3%	4042 ± 6.8%	54.8 ± 5.8%	474
9	1.55 X 10^5 ± 6.1%	2269 ± 6.7%	65.4 ± 11.1%	446
11	1.49 X 10^5 ± 7.9%	2669 ± 4.3%	234.0 ± 8.5%	222
13	0.83 X 10^5 ± 5.9%	761 ± 7.1%	122.1 ± 5.6%	193
15	0.77 X 10^5 ± 4.4%	1147 ± 8.1%	45.7 ± 6.0%	474
17	1.15 X 10^5 ± 2.9%	852 ± 2.6%	70.9 ± 2.5%	237
19	0.75 X 10^5 ± 5.9%	408 ± 3.2%	47.8 ± 11.3%	214
21	0.34 X 10^5 ± 2.7%	139 ± 3.5%	15.7 ± 2.0%	150
23	0.17 X 10^5 ± 3.8%	87 ± 2.3%	46.6 ± 1.5%	65
25	0.17 X 10^5 ± 2.2%	156 ± 1.9%	7.6 ± 1.4%.	180

Total percent ^{14}C incorporation into MeOH fraction = 0.09

Total percent ^{14}C incorporation into PHT = 0.22

TABLE XXV. ^{14}C UPTAKE INTO MEOH AND PE FRACTIONS OF *B. ALBA* LEAVES IN 24 HOURS*

ANOVA: for dpm MeOH/gFW

Source	D.F.	S.S.	M.S.	F value	F prob.	Tested against
TIME	12	8.34×10^{11}	6.95×10^{10}	1.8873	0.0851	2
SAMPLE	26	9.57×10^{11}	3.68×10^{10}	256.0845	0.0000	3
ALI	78	1.21×10^{10}	1.44×10^{8}			4
ERROR	0	0				
TOTAL	116	1.80×10^{12}				

ANOVA for dpm PE/gFW:

SOURCE	D.F.	S.S.	M.S.	F value	F prob.	Tested against
TIME	12	1.45×10^{9}	1.21×10^{8}	2.9323	0.0106	2
SAMPLE	26	1.07×10^{9}	4.12×10^{7}	63.6992	0.0000	3
ALI	78	5.05×10^{7}	6.47×10^{5}			4
ERROR	0	0				
TOTAL	116	2.57×10^{9}				

*Three-level nested design. Run #1.

TABLE XXVI. ^{14}C UPTAKE INTO PHT(3): *B. ALBA* LEAVES IN 24 HOURS*

ANOVA for dpm/mg (3):

Source	D.F.	S.S.	M.S.	F value	F prob.	Tested against
TIME	12	138012	11501	2.5704	0.0214	2
SAMPLE	26	116337	4474			3
ERROR	0	0				
TOTAL	38	254349				

* Two-level nested design, Run# 2.

of precursor molecules from leaf to root. Roots were harvested and extracted for compound 1 at the end of the 12 hour and week long experiment and the data in Table XXVII shows substantial incorporation of ^{14}C into the acetylene.

ACCUMULATION AND DISTRIBUTION OF PHENYLHEPTATRIYNE IN *BIDENS ALBA* SEEDLINGS

Two-day to 24-day seedlings of *B. alba* were extracted for PHT in the leaves, hypocotyls and roots. The relative amounts of PHT in each sample was determined using HPLC and the results shown in Table XXVIII and Figures 12 and 13. Phenylheptatriyne was present in two day old seedlings and levels in leaves increased throughout the experimental period. Concentrations in the hypocotyls decreased after one week. The roots contained 100 times higher amounts of PHT than the aerial tissues initially although quantities began to decline after two weeks.

TABLE XXVII. ^{14}C-LABELLED ENE-TETRAYNE-ENE (1) IN ROOTS OF *BIDENS* GIVEN $^{14}CO_2$.

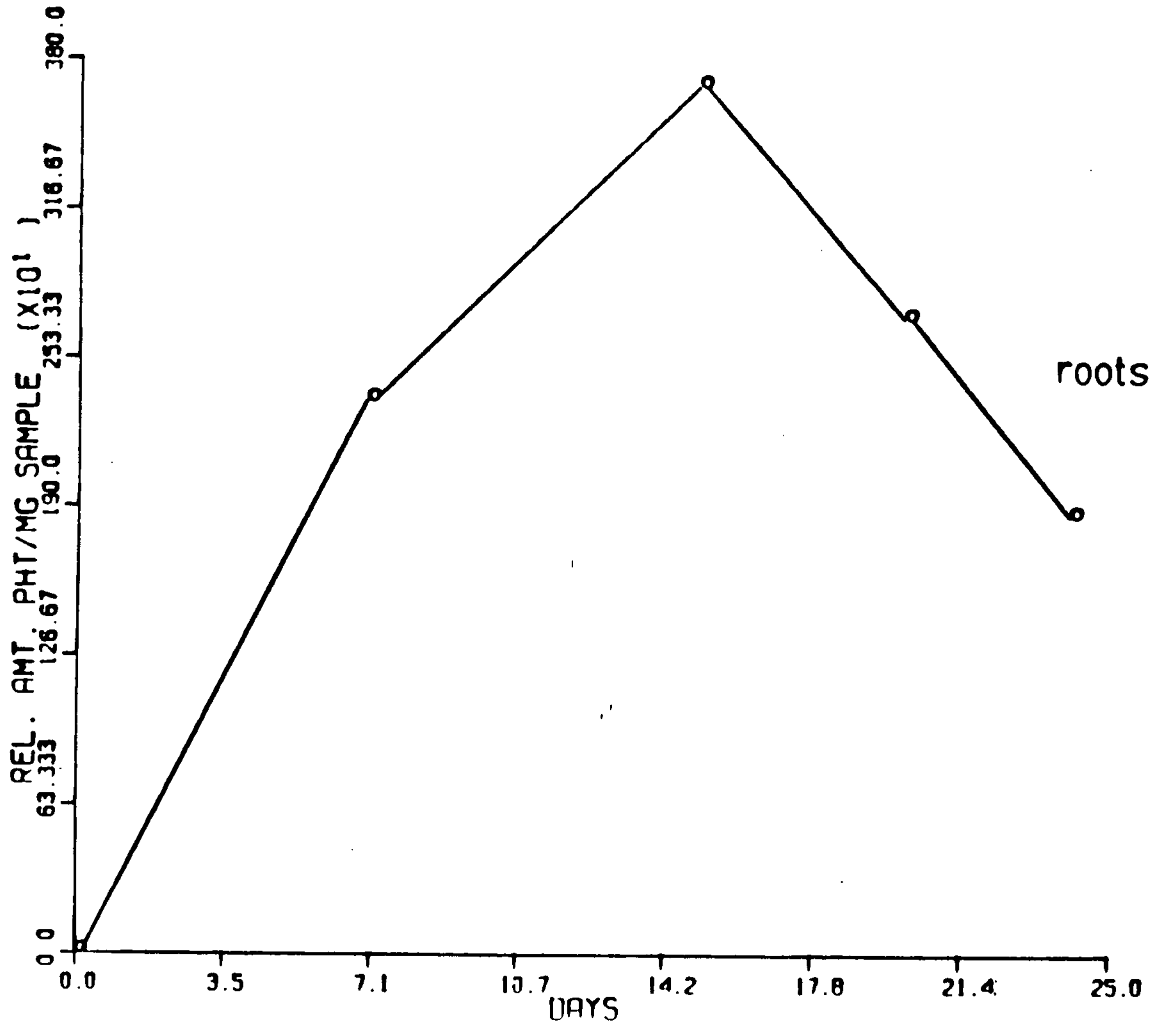
Time (hours)	Plant	1 dpm/mg	1 ug/gFW
13	*B. alba*	12078	9.0
13	*B. molokaiensis*	70000	0.1
13	*B. cosmoides*	268	11.3
13	*B. hillebrandiana*	25000	1.0
169	*B. alba*	2235	14.7
169	*B. molokaiensis*	6650	7.1
169	*B. cosmoides*	2692	2.7
169	*B. hillebrandiana*	4689	1.0

TABLE XXVIII. ACCUMULATION AND DISTRIBUTION OF PHENYLHEPTATRIYNE (4) IN *B. ALBA* SEEDLINGS

Days	FW mg/1000uL	mg/20uL sample	% area PHT/sample	Rel.amt. PHT/mg sample
Seedlings:				
2	122.4	2.45	49.31	20
4	125.3	2.51	77.05	31
5	100.8	2.02	76.88	39
Leaves:				
7	152.4	3.05	65.59	22
15	140.8	2.82	76.86	28
20	156.5	3.13	95.59	31
24	159.8	3.20	96.54	30
Hypocotyls:				
7	150.1	3.00	81.43	27
15	180.0	3.60	58.78	16
20	199.3	3.99	63.42	16
24	189.9	3.79	64.15	17
Roots:				
7	0.560	0.0112	26.37	2355
15	0.625	0.0125	46.17	3694
20	0.231	0.0046	12.46	2709
24	0.821	0.0164	30.64	1868

FIGURE 12. ACCUMULATION AND DISTRIBUTION OF PHT IN *BIDENS ALBA* SEEDLINGS

FIGURE 13. ACCUMULATION AND DISTRIBUTION OF PHT IN *BIDENS ALBA* SEEDLINGS



D. DISCUSSION

BIOSYNTHESIS OF POLYACETYLENES FROM $^{14}CO_2$ IN *BIDENS* LEAVES

The term biosynthesis is defined by Swain (1965) as the *in vivo* endothermic production of more complex molecules from simpler ones. Natural polyacetylenes are thought to be derived from fatty acid precursors which are systematically transformed into a dazzling array of compounds, some of which have potent photobiocidal effects (Bu'Lock, 1966; Bohlmann *et al.*, 1973; Jones and Thaller, 1978; Towers, 1979).

At present, no real evidence exists concerning the *in vivo* formation of the carbon-carbon triple bond although direct dehydrogenation via *cis* double bonds was favoured speculatively (Bu'Lock and Smith, 1967), and appears probable (Haigh *et al.*, 1968; Jones *et al.*, 1975; Jones and Thaller, 1978).

Nevertheless, the various biosynthetic interrelationships of acetylenes such as modifications of the chain lengths, introduction of triple bonds, rearrangements, introduction of oxygen- and sulphur-containing groups and cyclizations have been studied in great detail in the laboratories of F. Bohlmann and Sir Ewart R.H. Jones, mostly with the use of specifically labelled precursors obtained by total synthesis. These precursors include ^{14}C- and/or ^{3}H-labelled acetate, oleic and linoleic acids, crepenynic acid and dehydromatricaria ester variously administered via the intact root or leaf surface or the fungal culture medium (Bohlmann *et al.*, 1968; Bohlmann and Schulz, 1968; Fairbrother *et al.*, 1967; Jente and Richter, 1976; Jones, 1966).

Unlike the situation with microorganisms where putative precursors can be easily and naturally fed to a defined system, the uptake of labelled substances into plants is often difficult and unsatisfactory, the results not necessarily a true reflection of the *in vivo* situation (Swain, 1965; Brown and Wetter, 1972; Floss, 1977). The only way in which ^{14}C can be administered to plants in a physiological fashion is as $^{14}CO_2$ even though rates of incorporation into complex secondary metabolites may be rather low. This has not been previously demonstrated for polyacetylenes.

In the present study, the *de novo* biosynthesis of polyacetylenes in *Bidens* leaves was investigated in time course studies. ^{14}C-labelled polyacetylenes were recovered from three species of *Bidens* first administered $^{14}CO_2$ and subsequently allowed to metabolize in $^{12}CO_2$ for 12, 24 and 168 hours (Tables XVI to XXIV). In general, the plants incorporated 0.1 to 2.4 percent (mean = 0.9%) of the administered radioactivity into the methanol fraction from which 0.1 to 12.9 percent (mean = 2.9%) went into the polyacetylenes examined (Table XV). The range in values may be due to individual and/or interspecific variations in photosynthetic rates since environmental factors (CO_2

concentrations, H_2O, light and temperature) were essentially uniform for all experiments. These differences are most pronounced between *B. alba* and *B. molokaienses* in the 12 hour experiments. The *B. molokaiensis* plants available were older and evidently less vigorous than *B. alba* plants.

In all experiments, peak specific activity in the methanol fractions preceded that in the light petroleum fractions where most of the lipophilic compounds, including polyacetylenes, are found. Peak activity of polyacetylenes generally coincided with that of the light petroleum fractions. Specific activities of these components would be expected to rise initially with increasing formation from $^{14}CO_2$-derived intermediates and then fall as flushing with $^{12}CO_2$ proceeded.

Total amounts of polyacetylenes isolated at each time period are expressed as micrograms per gram fresh weight of leaves. This information suggests that PHT (4) and the ene-tetrayne-ene (1) are synthesized at a relatively constant rate in *B. alba* and *B. hillebrandiana* leaves, respectively (Tables XVI, XVII and XX, XXI). Levels of acetylenes remain essentially uniform while specific activity fluctuates. *Bidens cosmoides* leaves synthesize compounds 1, 3 and 5 but only 1 and 5 were easily detected in the system used here (TLC purification). The data for acetylene levels in *B. cosmoides* is inconclusive and may be due to the exclusion of the pentayne-ene from the analysis.

According to the scheme in Figure 7, compounds 1 and 5 are synthesized in parallel but separate pathways; one does not precede the other along the same sequence of reactions. In *B. cosmoides*, maximum specific activities of the two compounds occurs at the same time and decreases similarly. Either conversion from one acetylene to the other is extremely rapid or both are synthesized concurrently. Compound 3 was not investigated in these experiments but a comparison of its ^{14}C-uptake rate with that of 1 and 5 may have been more enlightening.

The highest specific activity of ^{14}C-PHT synthesized by *B. alba* was 0.0275 μCi/mg PHT (6.25 X 10^4 dpm/mg), 12 hours after 300 μCi of $^{14}CO_2$ was administered (Table XVI and Figure 14). In the 24 hour experiments, 500 μCi of $^{14}CO_2$ was fed to the plants and peak activity was even lower (0.0001 μCi/mg PHT or 234 dpm/mg) (Table XXIV) while that for the one week experiments was 0.0105 μCi/mg PHT or 2.38 X 10^4 dpm/mg (Table XX).

These results reflect the differences in total ^{14}C incorporated into the plants during each of the experiments (2.0%, 0.1% and 0.2%) and illustrate the difficulty of controlling the actual dose of ^{14}C fixed photosynthetically by whole plants. Other factors contributing to the variance may include relative differences in the metabolic activity of the biosynthetic sites as well as differences in the pool sizes of the acetylene precursors, postulated intermediates and those of the final products. In any case it appears that the natural synthesis of ^{14}C-labelled PHT with a significant amount of radioactivity may not be possible using this method.

At the end of the 12 hour and 168 hour experiments, roots were extracted for polyacetylenes. ^{14}C-labelled ene-tetrayne-ene (1) was detected in all plants (Table XXVII), including *B. molokaiensis*, which incorporated ^{14}C into its MeOH and PE fractions (Tables XIX, XXIII), in spite of the fact that it does not synthesize leaf acetylenes. This indicates that ^{14}C-labelled precursors were translocated from aerial tissues to sites in the roots where *de novo* synthesis of root compounds takes place.

ACCUMULATION AND DISTRIBUTION OF PHENYLHEPTATRIYNE IN *BIDENS ALBA* SEEDLINGS

In mature *B. alba* plants, leaves contain mainly PHT (**4**) while the stems have comparable amounts of PHT and phenylhepta-diyne-ene (**5**) and the roots **5** as well as the ene-tetrayne-ene (1) (Norton, 1984). The accumulation of polyacetylenes in developing *B. alba* plants has not been previously reported.

Detectable levels of PHT were found in two-day old seedlings of *B. alba*, suggesting that polyacetylene biosynthesis begins during germination or soon thereafter (Table XXVII and Figures 12, 13). Quantities in the leaves continue to increase up to 24 days and presumably beyond that to adult levels while amounts in the hypocotyls peak at seven days and subsequently decline to a lower concentration. This is probably accompanied by a concomitant increase in the levels of compound 5 (Figure 12).

PHT is absent from the roots of mature *Bidens alba* plants (Towers, 1980; Norton, 1984) but is present in the seedlings. Relative PHT levels in the roots are 100 times higher than those in the aerial tissues for the first 24 days. Nevertheless, there is also a gradual decline in these levels beginning at two weeks and continuing beyond the experimental time period (Table XXVII and Figure 13). This is accompanied by a concomitant increase in levels of compounds 1 and 5 (data not shown). It appears that the distribution of PHT in *B. alba* reaches its adult proportions by one month after the onset of germination.

E. CONCLUSION

The complete elucidation of a biosynthetic pathway requires the application of several different techniques. According to Adelberg (1953), these include isotopic labelling with precursors, *in vitro* enzyme studies and the use of microorganisms with blocked synthetic pathways. The main source of current knowledge of the pathways of acetylene biosynthesis are experiments of the first category (e.g., Bu'Lock, and Smith, 1963; Jones, 1966; Bohlmann *et al*., 1968).

In fungal cultures, biosynthetic experiments are easier and give higher incorporations than those with plants, although a variety of alternative sequences may be available for both types of organisms (Jones and Thaller, 1978).

In this study, the *de novo* biosynthesis of polyacetylenes in the leaves of selected species of Hawaiian *Bidens* and *Bidens alba* was demonstrated using $^{14}CO_2$. Levels of ^{14}C incorporated into the final products were minimal but all three acetylenes isolated were significantly labelled.

The validity of postulated biosynthetic sequences must be tested by more than one method, and ideally should be confirmed by the detection and isolation of enzymes catalyzing key steps in the pathway. Hawaiian species of *Bidens* should be useful organisms for *in vitro* studies for several reasons: they produce a limited array of acetylenes which are closely related, different species produce different arrays in leaves and roots and may be selected for particular compounds, interspecific hybrids are easily produced and the plants are relatively easy to propagate and maintain under standard greenhouse conditions.

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IV. PHOTOTOXICITY OF POLYACETYLENES TO PHYLLOPLANE FUNGI

A. INTRODUCTION

All Hawaiian *Bidens* possess acetylenes in the roots but only 15 taxa synthesize them in the leaves (Marchant *et al*.,1984). There appears to be no significant correlation between the presence or absence of leaf acetylenes and any other feature of *Bidens*, including habitat (F.R.Ganders, pers. comm.). Nearly all rainforest species (e.g., *B. cosmoides* and *B. macrocarpa*) have leaf acetylenes, but species with and without leaf acetylenes occur in drier habitats and lower elevations. Since morphological, genetic and biochemical data suggest that all native Hawaiian *Bidens* have evolved from a common ancestor (Ganders and Nagata, 1983a; 1983b; 1984; Helenurm and Ganders, 1985; Marchant *et al*.,1984), the genetic information for *de novo* acetylene synthesis in leaves and roots is probably present in *Bidens* but is not expressed in the leaves of certain taxa.

This phenomenon is not unique to Hawaiian *Bidens* species or even to *Bidens* in general. *Bidens cernua* L. (Bohlmann *et al*.,1973), *B.tripartita* L. (Bohlmann *et al*.,1962), *B.pilosa* var. *minor* (Blume) Sherff (Norton, 1984), as well as other species of Compositae (e.g., *Artemesia vulgaris* L., *Chrysanthemum douglasii* Hulten) (Bohlmann *et al*.,1973) do not synthesize leaf acetylenes, although they do so in the roots. This may be merely fortuitous.

Nevertheless many polyacetylenes are powerful photosensitizers (Towers, 1980) and are toxic to a wide range of organisms, including bacteria and fungi (Arnason *et al.*,1980; Camm *et al.*,1975; Chan *et al.*,1975; DiCosmo *et al.*,1982; Towers *et al.*,1977; Wat *et al.*,1977). This information has led to speculation about the possible biological significance of polyacetylenes in plants. Do these compounds have a specific function or set of functions in their parent plants? Does their presence signify a defense strategy against specific enemies or against all potentially threatening organisms? If so, why are they absent from the leaves of some species of *Bidens*? Do bioassays carried out on typical laboratory organisms such as *Escherichia coli* (Migula) Cast. et Chalm. and *Candida albicans* (Robin) Berkh. have relevance to the ecological conditions faced by *Bidens cosmoides* or other species growing in the wet jungle of Kauai? The present study is a preliminary attempt to answer some of these questions.

The aerial surfaces of higher plants growing under natural conditions are usually colonized by large and varied populations of microorganisms. Such populations colonizing leaf surfaces form an ecological niche which is termed the phylloplane (Last and Price, 1969; Davenport, 1976; Dickinson, 1976). Comparisons of the fungal populations on the leaves of different plants have led some authors to consider that one surface is very like another (DiMenna, 1971; Ruscoe, 1971), and although the majority of phylloplane studies have been carried out in temperate regions and on agricultural crops, the few studies which have hitherto been published on tropical plants suggest that many phylloplane fungi are cosmopolitan in distribution (Dickinson, 1976). These fungi grow on a wide range of host plants and populations, and species of fungi, particularly in temperate regions, are very similar (Last and Deighton, 1965). Nevertheless, Lamb and Brown (1971) examined *Paspalum dilatatum* L. (Poaceae), *Salix babylonica* L. (Salicaceae) and *Eucalyptus stellulata* Sieb. ex DC.(Myrtaceae) growing together on a creek bank and found that the leaves of these plants support distinctive microbial populations. They suggest that the phylloplane is a niche which is unfavourable for many of the organisms present in the airborne flora and that the leaf exerts selective pressure which determines the nature of the resident phylloplane microflora after the inoculum has come into contact with it.

One of the objectives of this study was to isolate and identify the phylloplane yeasts and yeast-like fungi on species of Hawaiian *Bidens* and on selected sympatric plants. The second purpose was to find out whether the occurrence and distribution of phylloplane fungi was correlated with the presence or absence of leaf acetylenes in *Bidens* . Finally, the sensitivity of these organisms to polyacetylenes was assessed and the biological role of acetylenes in leaves was evaluated.

B. MATERIALS AND METHODS

PLANT MATERIAL

Healthy green leaves from 12 taxa of Hawaiian *Bidens* and 23 sympatric species of other plants were collected in August, 1983 and February, 1984, placed in paper envelopes, sealed and allowed to dry during air transfer from Hawaii to Vancouver, Canada (Tables XXIX and XXX).

ISOLATION AND IDENTIFICATION OF FUNGI

Three methods were used to isolate phylloplane yeasts and yeast-like fungi from all the leaves: the spore fall method (Last, 1955), the leaf impression method (Potter, 1910; Lamb and Brown, 1970) and the leaf disc method (Petrini *et al.*, 1982; Carroll and Carroll, 1978). All isolates were cultured in a malt extract medium with tetracycline (MYPT), (Bandoni, 1972), (Table XXXI).

In the spore fall method (A), the dried leaves were first rehydrated by soaking in sterile water for 30 minutes, then placed in a plastic bag with a wad of moistened tissue paper, sealed and kept thus for 24 hours. Whole leaves were then rinsed in sterile water, dried and attached to the lids of sterile petri dishes, abaxial or adaxial surfaces exposed to the MYPT agar below. This method is a selective one which favours the isolation of members of the Sporobolomycetaceae by allowing their ballistospores to drop from leaf samples onto the nutrient medium.

TABLE XXIX. *BIDENS* TAXA SAMPLED FOR PHYLLOPLANE FUNGI

Taxa	Localities	Site descriptions
B. amplectens	Manini Pali, Oahu	Sunny ledges on steep cliffs about 200m elevation on windward side, north end of the Waianae Range, in scrub vegetation of *Lucaena leucocephala*, *Canthium odoratum*, *Myoporium sandwicense* and *Sida fallax*
B. cervicata	Makaha Ridge, Kauai	On roadside and open areas on dry ridge in planted pine forest; area rainfall about 40" per year; with native shrubs such as *Dodonaea* and *Styphelia*; elevation 500-700m.
B. cosmoides	Kokee State Park near Waimea Canyon overlook, Kauai	Rainforest of *Acacia koa*, *Metrosideros* with *Cyanea* and other plants; about 1000m elevation.
B. forbesii ssp. *forbesii*	Lumahai Beach overlook, Kauai	20-30m elevation on steep coastal bluffs above beach; in wet (75-100" rain per year) but rather scrubby vegetation with *Pandanus* and *Metrosideros*.

B. hawaiensis	Graveyard near Kaimu, Hawaii	Open mesic *Metrosideros* forests and open fields on old a'a lava flows; about 30m elevation; rainfall about 75" per year.
B. hillebrandiana ssp. *polycephala*	West side of Maliko Bay, Maui	On top of sea cliffs, 30-40m elevation; on windward side of island, exposed to ocean spray; bare soil between plants on the vertical cliff face and introduced weeds on top.
B. mauiensis	Near Chinese cemetery, Waihee, Maui	On windy, exposed, dry lithified sand dunes on the windward coast of west Maui; elevation 50m; with vegetation of native low shrubs and herbs; rainfall about 30"
B. menziesii ssp. *filiformis*	Ahumoa, Hawaii	On leeward slope of Mauna Kea, in open scrub and open dry forests of *Sophora* and *Myoporum* on the cinder cone of Ahumoa; about 2000m elevation; rainfall about 30" per year.

B. micrantha ssp. *ctenophylla*	Old quarry near Kailua, Kona, Hawaii	At about 30m elevation on ancient a'a lava flow; in arid, open scrub of *Schinus*, *Waltheria*, *Sida* and grasses; very low rainfall, about 10-15" per year.
B. populifolia	South ridge bordering Kahana Valley, Oahu	In clearings on ridge about 70-100m elevation; wet *Metrosideros* forest with *Pandanus*, *Schinus* and *Schefflera*
B. sandvicensis ssp. *confusa*	Iliau nature trail, Waimea Canyon, Kauai	*In open scrubland with Wilkesia*, *Dodonaea* and *Styphelia*; elevation about 700m; in open mesic forests of *Acacia koa* and *Metrosideros* at about 900m elevation.
B. sandvicensis ssp. *sandvicensis*	Waahila Ridge, Oahu	On exposed crest of ridge in mesic scrub vegetation; with *Sida*, *Osteomeles* and *Acacia koa*; about 400m elevation.
	Waimea Canyon, Kauai	About 350m elevation; in dry scrub of *Dodonaea* and introduced shrubs and herbs.

TABLE XXX. PLANTS ASSOCIATED WITH *BIDENS* SAMPLED FOR PHYLLOPLANE FUNGI

Bidens	Associated Plant Species
B. amplectens	*Canthium odoratum* Forst. f.
	Sida fallax Walp.
	Ilima sp.
	Myoporum sandwicense A. Gray
B. cervicata	*Styphelia tamehameha* (Cham.) F.Muell.
	Dodonaea sp.
	Lantana camara
B. cosmoides	*Psychotria* sp.
	Passiflora sp.
	Acacia koa A. Gray
B. forbesii ssp. *forbesii*	*Ageratum* sp.
	Metrosideros collina (Forst) Gray
	Stachytarpheta jamaicensis (L.)Vahl.
	Bidens pilosa L.
B. hillebrandiana ssp. *polycephala*	*Nicotiana glauca* Grah.
	Emilia sp.
	Trifolium sp.

B. mauiensis	*Lipochaeta* sp.
	Stachytarpheta jamaicensis (L.)Vahl.
	Ilima sp.
	Sida fallax Walp.
	Scaveola taccada (Gaertn.) Roxb.
	Waltheria americana L.
	Nama sandwicensis A.Gray
B. populifolia	*Stachytarpheta jamaicensis* (L.)Vahl.
	Passiflora sp.
	Osteomeles anthyllidifolia Lindl.
	Euphorbia sp.
	Wikstroemia sp.
B. sandvicensis ssp. *confusa*	*Styphelia tamehameha* (Cham.) F. Muell.
	Dodonaea sp.
	Wilkesia gymnoxiphium A.Gray
	Waltheria americana L.
	Lantana camara L.
B. sandvicensis ssp. *sandvicensis*	*Passiflora* sp.
	Acacia koa A. Gray
	Stachytarpheta jamaicensis (L.)Vahl.
	Sida fallax Walp.
	Osteomeles anthyllidifolia Lindl.

TABLE XXXI. COMPOSITION OF MALT EXTRACT (MYPT) CULTURE MEDIUM

Ingredients	grams/litre
malt extract	7.0
yeast extract	0.5
soytone	10.0
bacto agar	15.0
tetracycline HCl	0.05

The leaf impression method (B) was first described by Potter (1910) who used this technique to demonstrate the presence of fungi and bacteria on *Solanum* and *Helianthus* leaves. Since then, this method has been regularly used to provide data on the readily detachable component of the phylloplane saprophyte population. It provides an indication of the frequencies of both resident and transient fungal populations. Whole leaves were washed in sterile water and dried. Each leaf was placed, adaxial or abaxial surface up, on the agar surface, lightly pressed on the medium and subsequently removed.

The leaf disc method (C) was used to isolate endophytic organisms. It was modified from the methods described for conifer needles by Petrini *et al*. (1982) and Carroll and Carroll (1978). Leaves were washed in sterile water, then dipped briefly in 50% EtOH and transferred to a solution of 3.5% NaOCl:H_2O (1:9) for one minute. After a final rinse in sterile water, several discs of 10 mm diameter were cut from each leaf and transferred to the agar surface, covered and allowed to incubate.

In all three methods, prepared plates were incubated at 23°C (12 hour light/dark cycle) for up to 48 hours. Yeast and yeast-like fungal colonies were selectively transferred to new agar plates until pure cultures were obtained. Cultures are kept refrigerated at 4°C and transferred every two months.

The fungal isolates were identified by the Centraalbureau voor Schimmelcultures, P.O. Box 273, Oosterstraat 1, 3470 AG Baarn, Netherlands.

PHOTOTOXICITY ASSAYS

The photosensitivity of selected test organisms to the crude light petroleum extracts of Hawaiian *Bidens* leaves and that of isolated Hawaiian phylloplane fungi to several polyacetylenes was assayed using the method of Daniels (1965). Phylloplane fungi tested are listed in Table XXXII. Other test organisms included the yeasts *Saccharomyces cerevisiae* and *Candida albicans* , the gram positive bacteria *Staphylococcus albus* , *Streptococcus faecalis* and *Bacillus subtilis* , and the gram negative bacteria *Escherischia coli*, , *Pseudomonas fluorescens* and *P. aeuroginosa*. Cultures of bacteria and yeasts were obtained from the UBC culture collection. Sabouraud's medium was used for the yeasts and nutrient agar plates for the bacteria. All phylloplane fungi were cultured on MYPT agar plates.

Forty-eight hour liquid cultures in MYPT were streaked on agar plates with sterile cotton swabs. Light petroleum fractions and test compounds were dissolved in 95% EtOH at concentrations of 0.2 mg/mL, 1.0 mg/mL and/or 2 mg/mL. Five microlitres of each solution (i.e., 1 μg/disc, 5 μg/disc and 10 μg/disc) were applied to paper discs, 7mm in diameter, prepared from Whatman No.1 filter paper and the solvent allowed to dry in the dark. 8-Methoxypsoralen was used as a

TABLE XXXII. YEASTS AND YEAST-LIKE FUNGI ISOLATED FROM HAWAIIAN PLANTS

Class Basidiomycetes	
Sporobolomycetaceae	*Bullera* sp.
	Rhodotorula graminis di Menna
	R. mucilaginosa (Joerg.) Harrison
	R. pallida Lodder
	Sporobolomyces salmonicolor (Fischer et Brebeck) Kluyver et van Niel
	S. shibatanus (Okunuki) Verona et Ciferri
	S. roseus Kluyver et van Niel
	Tilletiopsis pallescens Gokhale
Cryptococcaceae	*Cryptococcus albidus* (Saito) Skinner
	C. laurentii (Kuff) Skinner
	C. luteolus (Saito) Skinner
Class Fungi Imperfecti	*Alternaria tenuissima* (Kze:Fr.) Wiltsch
	Aureobasidium pullulans (de Bary) Arnaud
	Cladosporium cladosporiodes (Fres.) de Vries
	C. cf. *cladosporiodes* (yellow pigment)

Class Fungi Imperfecti	*Colletotrichum gloeosporiodes* (Penzig) Penzig et Sacc.
	Epicoccum purpurescens Ehrenb.
	Hyphozyma variabilis de Hoog et M. Th. Smith
	Phoma sorghina (Sacc.) Boerema et al

reference photoactive compound (Fowlks *et al.*, 1958). The discs were placed on the inoculated plates which were prepared in duplicate. Test plates were exposed to longwave UV-A light (320-400nm) in a Psycrotherm incubator for two hours. Four Sylvannia black lights, F20T12-BLB, with irradiance of 10 watts/m^2 at 10 cm from source, measured with a YSI-Kettering Model 65 radiometer, were used. The controls were kept in the dark. All phylloplane organisms were subsequently incubated at 23°C for 48 hours. *Saccharomyces*, *Candida* and bacterial species were incubated at 37°C.

Compounds which produced zones of inhibition of microbial growth only upon irradiation are phototoxic. Those samples which gave similar sizes of zones of inhibition both in light and dark are antibiotic. All assays were repeated three to five times and the results combined.

COMPARISON OF PHOTOTOXICITY OF SELECTED POLYACETYLENES TO *CRYPTOCOCCUS LAURENTII*

A 48 hour stationary culture of *Cryptococcus laurentii* grown in Malt Yeast Peptone (MYP) broth at 23°C was diluted to approximately 1.69 X 10^4 cells/mL in fresh medium. One hundred microlitre aliquots of this suspension were added to wells of sterile microtitre plates (Nunc-96FB with lids) using a Titertek Multichannel pipette. A series of nine two-fold dilutions of phenylheptatriyne (PHT, compound **4**), phenyl-diyne-ene (compound **5**), heptadeca-tetraene-diyne (compound **8**) and α-terthienyl (compound **21**) were made with growth medium (MYP). Six replicates of 100uL of each dilution were added to the test wells containing yeasts.

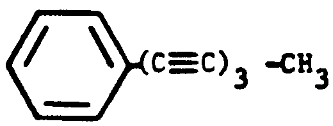
The starting concentrations were 5 μg/mL or 10 μg/mL with a maximum of 1% EtOH to avoid solvent toxicity. One row in each plate was set up as a control, with growth medium only. For each compound, one plate served as the dark control (0'), and test plates were irradiated with long wave UV (320-400nm) for 5,10 and 20 minutes. All plates were subsequently incubated at 23°C. The optical density (O.D.), at 492 nm, of each solution in each of the test wells was read before irradiation (T_0), and at 19 hours (T_{19}), 24 hours (T_{24}), 40 hours (T_{40}) and 48 hours (T_{48}) later.

TEST COMPOUNDS

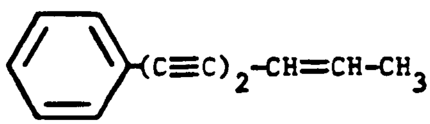
Crude light petroleum (BP 30-60°C) extracts and compounds **1**,**4**, **5**,**7**,**8** and **9** were prepared and isolated from selected species of Hawaiian *Bidens* according to the methods described in Chapter II (Table XXXIII). Compound **21**, α-terthienyl was a gift from Thor Arnason, University of Ottawa, compound **22** thiarubine A was isolated by Alex Finlayson from *Eriophyllum lanatum* (Pursh) Forbes (Norton *et al.*,1985). 8-Methoxypsoralen was obtained from Sigma Chemical. All compounds were dissolved in 95% EtOH at 0.2, 1.0 or 2.0 mg/ml.

TABLE XXXIII. POLYACETYLENES USED FOR PHOTOTOXICITY ASSAYS

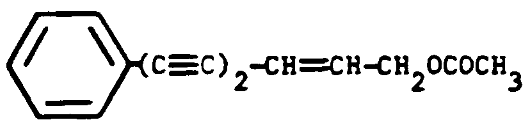
$CH_2{=}CH(C{\equiv}C)_4CH{=}CH{-}CH_3$ 1



4



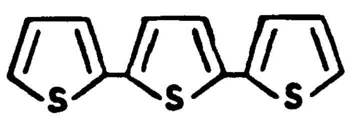
5



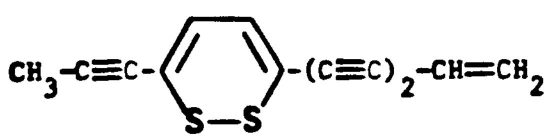
7

$CH_3{-}CH{=}CH(C{\equiv}C)_2(CH{=}CH)_2(CH_2)_4{-}CH{=}CH_2$ 8

$CH_3{-}(C{\equiv}C)_3(CH{=}CH)_2(CH_2)_4{-}CH{=}CH_2$ 9



21



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C. RESULTS

ISOLATION, IDENTIFICATION AND DISTRIBUTION OF PHYLLOPLANE ORGANISMS

Leaves from 12 species of *Bidens* and 23 species of associated tropical plants were collected from 13 different localities in Hawaii in August, 1983 and February, 1984 (Tables XXIX and XXX). Nineteen taxa of yeasts and yeast-like fungi belonging to the Sporobolomycetaceae, the Cryptococcaceae and the Fungi Imperfecti were selectively isolated and samples of isolates identified by the Centraalbureau voor Schimmelcultures (Table XXXII) (Kreger-van Rij, 1984; 1965; Barnett *et al.*, 1979). Three methods were used for the isolation of microorganisms, each one with its particular advantages and limitations (Potter, 1910; Last, 1955; Lamb and Brown, 1970; Carroll and Carroll, 1978). A comparison of the distribution of the seven most common genera isolated by each method is presented in Tables XXXIV to XXXVI.

On agar plates inoculated with dilutions or leaf washings, yeasts such as *Sporobolomyces* are usually overgrown by other fungal species. Separation can be effected by allowing these organisms to discharge their spores so that they fall on the culture medium (Last, 1955). The spore fall method also provides an indication of the actively growing and sporulating fungi on the surfaces of leaves. This is based on the assumption that spores produced

TABLE XXXIV. DISTRIBUTION OF FUNGI ISOLATED BY THE SPORE FALL METHOD

Plants	Sp	Rh	Cry	Clad	Epi	Aureo	Coll
Site 1: Maliko Bay, Maui							
B. hillebrandiana ssp. *polycephala*	-	-	+	-	-	-	-
Nicotiana glauca	-	-	-	+	-	-	-
Emilia sp.	++	-	++	+++	-	-	-
Trifolium sp.	-	-	+	-	-	-	-
Site 2: Kokee State Park, Kauai							
*B. cosmoides**	+	-	+++	+	-	-	-
Psychotria sp.	-	-	++	++	-	-	-
Passiflora sp.	-	-	-	-	-	-	-
Acacia koa	+	-	-	-	-	-	-
Site 3: Lumahai Beach, Kauai							
B. forbesii ssp. *forbesii*	-	-	-	++	-	-	-
Ageratum sp.	+	-	-	-	-	-	-
Metrosideros collina	-	-	+	+++	-	-	-
Stachytarpheta jamaicensis	-	-	-	++	-	-	-
B. pilosa	-	-	++	++	-	-	-
Site 4: Kohana Valley, Oahu							
B. populifolia	+	-	-	++	-	-	-
Stachytarpheta jamaicensis	-	-	+	++	-	-	-
Passiflora sp.	-	-	-	+	-	-	-
Osteomeles anthyllidifolia	-	-	-	-	-	-	-
Euphorbia sp.	-	-	+	+	-	-	-
Wikstroemia sp.	-	-	+	+	-	-	-

Site 5: Waimea Canyon, Kauai							
B. sandvicensis ssp. *confusa*	-	-	+	++	-	-	-
Styphelia tamehameha	++	-	-	-	-	-	-
Dodonaea sp.	-	-	-	+++	-	-	--
Wilkesia gymnoxiphium	-	-	++	+	-	-	-
Waltheria americana	-	-	+	+	-	-	-
Lantana camara	-	-	-	++	-	-	-
Site 6: Waahila Ridge, Oahu							
B. sandvicensis ssp. *sandvicensis*	+	-	-	+	-	-	-
Passiflora sp.	+	-	-	-	-	-	-
Acacia koa sp.	-	-	-	-	-	-	-
Stachytarpheta jamaicensis	-	-	-	-	-	-	-
Sida fallax	+	-	++	-	-	-	-
Osteomeles anthyllidifolia	-	-	-	+	-	-	-
Site 7: Makaha Ridge, Kauai							
*B. cervicata***	++	-	++	+++	-	-	-
Styphelia tamehameha	-	-	-	-	-	-	-
Dodonea sp.	-	-	+	+++	-	-	-
Lantana camara	+	-	+	2+++	-	-	-
Site 8: Cemetery, Waihee, Maui							
B. mauiensis	+	-	++	2+++	-	+	-
Stachytarpheta jamaicensis	+	-	+	+++	-	-	-
Lipochaeta ssp.	-	-	-	+	-	-	-
Ilima ssp.	+	-	+	+	-	-	-
Scaveola taccada	-	-	-	++++	-	-	-
Waltheria americana	-	-	+	+	-	-	-

Site 9: Manini Pali, Oahu							
B. amplectens	-	-	-	-	-	-	-
Canthium odoratum	-	-	+	-	-	-	-
Ilima sp.	-	-	-	+	-	-	-
Myoporum sandwicense	-	-	-	+	-	-	-
Site 10: Ahumoa, Hawaii							
B. menziesii ssp. *filiformis*	-	-	-	+++	-	-	--

+ less than 4 colonies/plate

++ 4-8 colonies/plate

+++ greater than 8 colonies/plate

++++ covers entire plate

* also isolated *Bullera* sp., *Hyphomycetes* sp.

** also isolated *Tilletiopsis pallescens* by actively growing fungi are more likely to be liberated and fall to the nutritive agar surface under the action of gravity than spores which are present by chance and merely adhere to the leaf surface (Lamb and Brown, 1970).

Several species of *Sporobolomyces* and *Cryptococcus* were isolated with this method (Table XXXIV). *Bullera* and *Tilletiopsis pallescens* Golkhale were detected once, from the leaves of *B. cosmoides*, whereas *Cladosporium cladosporiodes* (Fres.) deVries was frequently isolated this way, probably because its spore discharge is affected by humidity changes (Dickinson, 1971). In general, *Cladosporium* appears to be the most commonly occurring species found in this study, being present on leaves of all plants from all localities sampled by each method (Tables XXXIV to XXXVI). *Tilletiopsis*, a mycelial genus, is reported to be regularly isolated from leaves (Last, 1955; 1970; Ruscoe, 1971; Pady, 1974; Dickinson, 1976). Its rare occurrence in this study may be because of two reasons. It appears to require a higher growth temperature than *Sporobolomyces* and it grows more slowly on laboratory culture media so that it may be easily overlooked in spore fall isolation plates (Dickinson, 1976; Last and Price, 1969).

If a species is detected by the spore fall method, it is likely to be an active saprophyte and therefore sporulating on the leaf surface. It therefore should also be isolated by the leaf impression method. This correlation is demonstrated by the data in Table XXXV where the presence of

TABLE XXXV. DISTRIBUTION OF FUNGI ISOLATED WITH THE LEAF IMPRESSION METHOD

Plants	Sp	Rh	Cry	Clad	Epi	Aureo	Coll
Site 1: Maliko Bay, Maui							
B. hillebrandiana ssp. *polycephala*	+	+	-	++	-	-	+++
Nicotiana glauca	+	+	++	2++++	-	-	-
Emilia sp.	+	+	++	++++	-	+	-
Trifolium sp.	+	+	+++	2++++	-	+	-
Site 2: Kokee State Park, Kauai							
B. cosmoides	+++	+++	++	++++	+	-	-
Psychotria sp.	++++	++++	+	++++	-	-	-
Passiflora sp.	+++	+++	++	++++	-	-	+
Acacia koa	+++	+++	++	++++	-	-	-
Site 3: Lumahai Beach, Kauai							
B. forbesii ssp. *forbesii*	+	+	+	+++	+	-	-
Ageratum sp.	++	++	++	+++	+	-	-
Metrosideros collina	++	++	++	+++	-	-	-
Stachytarpheta jamaicensis	+	+	+	2+++	-	+	-
B. pilosa	++	++	+++	+++	-	-	++
Site 4: Kohana Valley, Oahu							
B. populifolia	-	-	-	+++	+	++	-
Stachytarpheta jamaicensis	-	-	-	++++	-	++	-
Passiflora sp.	++	++	-	++	-	-	+
Osteomeles anthyllidifolia	+	+	-	++	+	-	-
Euphorbia sp.	+	+	-	+++	-	-	-
Wikstroemia sp.	++	++	+	++	-	-	-

Site 5: Waimea Canyon, Kauai							
B. sandvicensis ssp. *confusa*	+	+	+	+++	++	++	-
Styphelia tamehameha	++	++	+	+++	+	++	-
Dodonaea sp.	++	++	-	+++	+	+	-
Wilkesia gymnoxiphium	++	++	++	++	-	+	-
Waltheria americana	+	+	-	++	-	+	-
Lantana camara	++	++	-	+++	++	-	-
Site 6: Waahila Ridge, Oahu							
B. sandvicensis ssp. *sandvicensis*	+	+	+	+++	++	+	-
Passiflora sp.	-	-	-	++	-	-	-
Acacia koa sp.	+	+	-	+	-	-	+
Stachytarpheta jamaicensis	+	+	++	+++	++	-	+
Sida fallax	-	-	++	++	-	-	-
Osteomeles anthyllidifolia	-	-	+	++++	-	-	+
Site 7: Makaha Ridge, Kauai							
B. cervicata	-	-	-	++++	++	-	-
Styphelia tamehameha	-	-	+	+++	-	-	-
Dodonaea sp.	-	-	+	++++	-	-	-
Lantana camara	-	-	+	++++	++	+	-
Site 8: Cemetery, Waihee, Maui							
B. mauiensis	-	-	+	2+++	-	-	++
Stachytarpheta jamaicensis	-	-	+	+++	-	+	-
Lipochaeta ssp.	+	+	+	+++	+	-	+
Ilima ssp.	+	+	-	++++	-	-	+
Scaveola taccada	-	-	+	+++	++	-	+
Waltheria americana	-	-	+	2+++	-	++	++

Site 9: Manini Pali, Oahu							
B. amplectens	+	+	++	+++	-	++	+
Canthium odoratum	+	+	+	+++	++	+	-
Ilima sp.	+	+	-	2+++	-	++	+
Myoporum sandwicense	+	+	+	2+++	-	++	+++
Site 10: Ahumoa, Hawaii							
B. menziesii ssp. *filiformis*	-	-	-	+++	++	-	-
Site 11: Kailua, Kona, Hawaii							
B. micrantha ssp. *ctenophylla*	-	-	-	-	-	++	-
Site 12: Kaimu, Hawaii							
B. hawaiensis	++	++	-	-	-	-	-

+ less than 4 colonies/plate

++ 4-8 colonies/plate

+++ greater than 8 colonies/plate

++++ covers entire plate

Sporobolomyces, *Cryptococcus* and *Cladosporium* species is recorded. *Rhodotorula* species and *Aureobasidium pullulans* (de Bary) Arnaud are also important primary leaf colonizers which adhere to the phylloplane surface (Last and Price, 1969; Pugh and Buckley, 1971), whereas *Epicoccum purpurescens* is believed to be a phylloplane invader which exhibits a pattern of restricted development until conditions on the leaf surface become particularly favourable (Dickinson, 1976). All three were isolated by the leaf impression method.

Colletotrichum gloeosporiodes (Penzig) Penzig et Sacc. is a pathogenic species which invades leaf tissue (Blakeman *et al*., 1971; Marks *et al*., 1965). It was isolated mainly from surface-sterilized leaves using the leaf disc method, (Table XXXVI) but was also found using the leaf impression method, presumably because the organism exists on the leaf before it enters the tissue (Blakeman, 1971; Marks *et al*., 1965). Species of *Cladosporium*, *Epicoccum* and, rarely, *Cryptococcus* and *Sporobolomyces* were also isolated from surface sterilized leaves (Table XXXVI). The first two genera have been reported to grow actively within the leaf and *C. cladosporiodes* forms microsclerotia which are able to withstand desiccation and probably other adverse environmental factors (Pugh and Buckley, 1971; Ruscoe, 1971; Dickinson, 1976).

One of the objectives of this study was to establish whether the occurrence and distribution of phylloplane fungi

TABLE XXXVI. DISTRIBUTION OF FUNGI ISOLATED WITH THE LEAF DISC METHOD

Plants	Sp	Rh	Cry	Clad	Epi	Aureo	Coll
Site 1: Maliko Bay, Maui							
B. hillebrandiana ssp. *polycephala*	-	-	-	++	-	-	+
Nicotiana glauca	-	-	-	-	-	-	+
Emilia sp.	+	+	-	++	-	-	-
Trifolium sp.	+	+	+	++	-	-	+
Site 2: Kokee State Park, Kauai							
B. cosmoides	-	-	-	++	+	-	-
Psychotria sp.	-	-	-	-	-	-	-
Passiflora sp.	-	-	-	-	-	-	+++
Acacia koa	-	-	-	-	-	-	-
Site 3: Lumahai Beach, Kauai							
B. forbesii ssp. *forbesii*	-	-	-	++	-	-	+
Ageratum sp.	-	-	-	++	-	-	++
Metrosideros collina	-	-	-	++	-	-	-
Stachytarpheta jamaicensis	-	-	-	++	-	-	+++
B. pilosa	-	-	-	++	-	-	++
Site 4: Kohana Valley, Oahu							
B. populifolia	-	-	-	-	-	-	+
Stachytarpheta jamaicensis	-	-	-	+	-	-	+
Passiflora sp.	-	-	-	++	+	-	+
Osteomeles anthyllidifolia	-	-	-	-	-	-	-
Euphorbia sp.	-	-	-	-	-	-	-
Wikstroemia sp.	-	-	-	+++	-	-	++

Site 5: Waimea Canyon, Kauai							
B. sandvicensis ssp. *confusa*	-	-	-	+++	-	-	-
Styphelia tamehameha	-	-	-	++	-	-	-
Dodonaea sp.	-	-	-	-	+	-	-
Wilkesia sp.	-	-	-	-	-	-	-
Waltheria americana	-	-	-	-	+	-	-
Lantana camara	-	-	-	-	-	-	-
Site 6: Waahila Ridge, Oahu							
B. sandvicensis ssp. *sandvicensis*	-	-	-	+	-	-	-
Passiflora sp.	-	-	-	-	-	-	-
Acacia koa	-	-	-	-	-	+	-
Stachytarpheta jamaicensis	-	-	-	-	-	-	-
Sida fallax	-	-	-	+	-	-	-
Osteomeles anthyllidifolia	-	-	-	-	-	-	-
Site 7: Makaha Ridge, Kauai							
B. cervicata	-	-	-	++	+	-	-
Styphelia tamehameha	-	-	-	-	-	-	-
Dodonaea sp.	-	-	-	-	+	-	+
Lantana camara	-	-	-	+++	-	-	-
Site 8: Cemetery, Waihee, Maui							
B. mauiensis	-	-	-	+	-	-	+
Stachytarpheta jamaicensis	-	-	-	+	-	-	+
Lipochaeta ssp.	-	-	+	++	-	-	-
Ilima ssp.	-	-	-	-	-	-	-
Scaveola sp.	-	-	-	++	-	-	-
Waltheria americana	-	-	-	++	-	-	-

Site 9: Manini Pali, Oahu							
B. amplectens	-	-	-	-	-	-	+
Canthium odoratum	-	-	-	-	+	-	-
Ilima sp.	-	-	+	+	-	-	++++
Myoporum sandwicense	-	-	+++	-	-	-	+
Site 12: Kaimu, Hawaii							
B. hawaiensis	-	-	-	-	-	-	++

+ less than 4 colonies/plate

++ 4-8 colonies/plate

+++ greater than 8 colonies/plate

++++ covers entire plate

is correlated with the presence or absence of leaf acetylenes in *Bidens*. The data shown in Table XXXVII indicates that at least non filamentous saprophytes do not seem to distinguish among *Bidens* species. Nevertheless, *Colletrotrichum* was found in only two of the five (40%) leaf acetylene producing *Bidens* species, and is absent from *Bidens* which produce the C_{13} aromatic compounds 5 and 7 (Tables III, IV). It was detected in five of seven (71.4%) *Bidens* species without acetylenes. In addition, *Aureobasidium pullulans*, a common phylloplane organism, was isolated from only one of the first group of *Bidens* (20%) and from three out of four species of the second.

All except three *Bidens* taxa were hosts to at least five of the seven fungi listed. *Bidens hawaiensis* and especially *B. menziesii* ssp. *filiformis* and *B. micrantha* ssp. *ctenophylla* were all collected from arid to semiarid exposed sites on the island of Hawaii (Table XXIX) which would be expected to have a lower diversity of fungal species than a typical rainforest locality such as that of *B. cosmoides* (Dickinson, 1967; 1976; Dickinson and O'Donnell, 1977; Ruinen, 1961).

PHOTOSENSITIVITY OF MICROORGANISMS TO ACETYLENES

The light petroleum fractions of leaf and root extracts from species of Hawaiian *Bidens* were tested for phototoxicity against nine species of fungi and bacteria

TABLE XXXVII. DISTRIBUTION OF YEASTS AND YEAST-LIKE FUNGI AMONG HAWAIIAN *BIDENS*

Bidens with leaf acetylenes	Sp	Rh	Cry	Clad	Epi	Aureo	Coll
B. hillebrandiana	+	+	+	+	-	-	+
B. cosmoides	+	+	+	+	+	-	-
B. cervicata	+	-	+	+	+	-	-
B. sandvicensis ssp. *confusa*	+	+	+	+	+	+	-
B. hawaiensis	+	+	-	-	-	-	+
Bidens without leaf acetylenes							
B. forbesii ssp. *forbesii*	+	+	+	+	+	-	+
B. mauiensis	+	-	+	+	-	+	+
B. amplectens	+	+	+	+	-	+	+
B. menziesii ssp. *filiformis*	-	-	-	+	+	-	-
B. populifolia	+	-	-	+	+	+	+
B. sandvicensis ssp. *sandvicensis*	+	+	+	+	+	+	+
B. micrantha ssp. *ctenophylla*	-	-	-	-	-	+	-

using the method of Daniels (1965). While the root samples were found to be consistently phototoxic as expected, only leaf extracts containing acetylenes were lethal to the test organisms in the presence of long wave UV light (Tables XXXVIII, XXXIX).

The yeasts *S. cerevisiae* and *C. albicans* were sensitive to most of the *Bidens* containing acetylenes, as were the gram-positive bacteria. The gram-negative bacteria were generally unaffected. This is in agreement with the data of Towers *et al.*, (1977). The most photoactive extracts were those from *Bidens* species which produce the C_{17} hydrocarbon compounds **8**, **9**, and **10** (*B. campylotheca*, ssp. *pentamera*, *B. conjuncta*, *B. macrocarpa*, *B. menziesii* ssp. *menziesii*, *B. hillebrandiana* ssp. *polycephala*, *B. torta* 17C) and/or C_{13} hydrocarbon and aromatic compounds **1**, **4**, **5** and **7** (*B. campylotheca* ssp. *pentamera*, *B. cosmoides*, *B. sandvicensis* ssp. *confusa*, *B. torta* 17A and 17B). *Bidens hawaiensis* with leaves containing compound **18** ("safynol") also exhibited phototoxicity against several of the test organisms.

8-Methoxypsoralen (8-MOP) was used as a reference test compound. It is a well known phototoxic furanocoumarin (Towers, 1980; Warren *et al.*, 1980; Averbeck, 1982; Ashwood-Smith *et al.*, 1980) and is lethal to the organisms used in this study except *Pseudomonas* sp. (Fowlks *et al.*, 1958.

Relative differences in the phototoxicity of test extracts can be quantified by measurement of the diameters

TABLE XXXVIII. PHOTOSENSITIVITY OF MICROORGANISMS TO EXTRACTS OF HAWAIIAN *BIDENS* LEAVES*

Microorganisms	8 MOP		3a		5		6		7		10		11		12		12a		13	
	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK
Sacch. cerevisiae	+++	-	++	-	+	-	++++	-	-	-	++	-	-	-	+	-	-	-	-	-
Candida albicans	+++	-	-	-	+	-	+++	-	-	-	++	-	-	-	+	-	-	-	-	-
Staphylccoccus aureus	+++	-	-	-	-	-	+	-	-	-	++	-	-	-	+	-	-	-	-	-
S. albus	+++	-	++	-	+	-	++	-	-	-	+++	-	-	-	++++	-	-	-	-	-
Streptococcus faecalis	+++	-	+++	-	+	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-
Bacillus subtilis	+++	-	+	-	+	-	++	-	-	-	++++	-	-	-	-	-	-	-	-	-
Escherichia coli	+++	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeuroginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* 10 ug/disc of light petroleum extracts; *Bidens* key in Table II

\+ clear zone diameter 7-10mm

++ clear zone diameter 10-14mm

+++ clear zone diameter 14-18mm

++++ clear zone diameter >> 18mm

TABLE XXXIX. PHOTOSENSITIVITY OF MICROORGANISMS TO EXTRACTS OF HAWAIIAN *BIDENS* LEAVES*

Microorganisms	8 MOP		9		16		16a		17A		17B		17C		18		8		15	
	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK
Saccharomyces cerevisiae	+++	-	++	-	-	-	+	-	+	-	+	-	++	-	+	-	+	-	-	-
Candida albicans	+++	-	++	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-	-	-
Staphylococcus aureus	+++	-	++	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-
S. albus	+++	-	+++	-	-	-	+	-	+	-	++	-	++++	-	+	-	++	-	-	-
Streptococcus faecalis	+++	-	++	-	-	-	+	-	-	-	+	-	+++	-	-	-	+	-	-	-
Bacillus subtilis	+++	-	+++	-	-	-	++	-	+	-	++	-	+	-	+	-	-	-	-	-
Escherichia coli	+++	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Pseudomonas fluorescens	-	-	++	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeuroginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* 10 ug/disc of light petroleum extracts; *Bidens* key in Table II

+ clear zone diameter of 7-10mm

++ clear zone diameter of 10-14mm

+++ clear zone diameter of 14-18mm

++++ clear zone diameter >> 18 mm

of the clear zones around the impregnated filter paper discs. The minimum measurable zone of inhibition is 7mm, the diameter of the disc. Clear zone diameters were assigned values on a scale of + (7-10mm) to ++++ (greater than 18mm) in order to demonstrate relative phototoxicities. Irradiation itself does not affect the growth of the organisms.

Thirteen species of yeasts and yeast-like fungi isolated from Hawaiian *Bidens* leaves were tested for photosensitivity to polyacetylenes from *Bidens* as well as to α-terthienyl (compound **21**) and thiarubrine A (compound **22**) from *Tagetes* and *Eriophyllum* species (Compositae) respectively (Tables XXXIII, XL and XLI). Concentrations of 1,5 and 10 μg/mL were used because leaf acetylenes in *Bidens* do not exceed 10 μg/mL. The C_{13} 'ene-tetrayne-ene' compound **1** was ineffective against all organisms except *Tilletiopsis pallescens* and *Colletotrichum gloeosporiodes*. The C_{17} compounds **8** and **9**, which were strongly photoactive against bacteria, *Saccharomyces* and *Candida* (Tables XXXVIII, XXXIX) were slightly toxic to *Cryptococcus* species but not at all deleterious to their pigmented relatives *Rhodotorula* sp. or to members of the Sporobolomycetaceae and Fungi Imperfeci.

The C_{13} aromatic compounds **4**,**5** and **7**, the thiophenes **21** and **22**, as well as 8-methoxypsoralen were phototoxic to most of the phylloplane fungi. The thiophenes **21** and **22** were used in this study because they are known to be powerful photosensitizers (Towers, 1979; Downum *et al*., 1982). They

TABLE XL. PHOTOSENSITIVITY OF *CR. LAURENTII* FROM DIFFERENT HOST PLANTS TO POLYACETYLENES

Host Plants	8 MOP		1		5		7		8		9		4		21		22	
	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK
B. cosmoides	+	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+
B. hillibrandiana ssp. *polycephala*	+	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+
B. mauiensis	+	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+
B. sandvicensis ssp. *sandvicensis*	+	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+
B. sandvicensis ssp. *confusa*	+	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+

* 1-5 ug/disc; see Tables III and XXXIII

\+ clear zone diameter of 7-10mm

++ clear zone diameter of 10-14mm

+++ clear zone diameter of 14-18mm

++++ clear zone diameter >>18 mm

TABLE XLI. PHOTOSENSITIVITY OF PHYLLOPLANE FUNGI TO POLYACETYLENES*

Microorganisms	8 MOP		1		5		7		8		9		4		21		22	
	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK	UV	DK
Sporobolomyces roseus	-	-	-	-	-	-	-	-	-	-	-	-	++	-	++	-	++	+
S. shibatanus	+++	-	-	-	++	-	+	-	-	-	-	-	++++	-	+++	-	++	+
S. salmonicolor	++	-	-	-	-	-	++	-	-	-	-	-	+++	-	++	-	++++	++++
Tilletiopsis pallescens	++	-	+	-	+++	-	NT	NT	-	-	-	-	NT	NT	++++	-	NT	NT
Rhodotorula pallida	++	-	-	-	-	-	-	-	-	-	-	-	++	-	++	-	++++	++++
R. mucilaginosa	+	-	-	-	++++	++	+++	++	-	-	-	-	+	-	-	-	++++	-
Cryptococcus albidus	+	-	-	-	+	-	+	-	+	-	+	-	++	-	+	-	++	++
C. laurentii	++	-	-	-	+	-	+	-	+	-	+	-	+++	-	++	-	++	+
C. luteolus	+	-	-	-	+	-	NT	NT	+	-	+	-	+	-	++++	-	NT	NT
Cladosporium cladosporiodes	++	-	-	-	++	++	++	-	-	-	-	-	++++	-	+++	-	+++	++++
Epicoccum purpurescens	++	-	-	-	+	+	-	-	-	-	-	-	-	-	+++	-	+++	++++
Aureobasidium pullulans	+	-	-	-	+	-	+	-	-	-	-	-	+	-	++	-	+	+
C. gloeosporiodes	+	-	++++	++	++++	-	NT	NT	-	-	-	-	NT	NT	++++	-	NT	NT

* 1-5 ug/disc; see Tables III and XXXIII

+ clear zone diameter of 7-10mm

++ clear zone diameter of 10-14 mm

+++ clear zone diameter of 14-18mm

++++ clear zone diameter >> 18 mm

NT not tested

were effective against all the organisms tested except for *Rhodotorula mucilaginosa* (Joerg.) Harrison which was resistant to α-terthienyl and not inhibited by thiarubrine A in the dark. Except for one population (17A) of *B. torta*, compound **4** (PHT) does not occur in Hawaiian *Bidens*. It is also deleterious to the majority of fungi tested. 8-Methoxypsoralen, used as a reference photoactive compound, was effective against all but *Sporobolomyces roseus* Kluyver et van Niel. This organism is also resistant to five other test acetylenes and relatively insensitive to α-terthienyl and thiarubrine A. The pathogenic endophyte *Colletotrichum gloeosporiodes* is very sensitive to compound **1** both in the light and in the dark. In addition, it is easily killed by compound **5**. This organism was absent from leaves of *Bidens* containing aromatic acetylene **5** (Table XXXVII) although present in *Bidens* containing other acetylenes. It is resistant to compounds **8** and **9**.

Aureobasidium pullulans, isolated from only one of five *Bidens* species producing leaf acetylenes, *B. sandvicensis* ssp. *confusa* (Table XXXVII), is only slightly photosensitive to compounds **5** and **7** which occur in the host plant and to the closely related compound **4**. It is also least sensitive of all organisms tested against thiarubrine A. *Epicoccum purpurescens* is insensitive to all *Bidens* compounds except compound **5**. *Cladosporium cladosporiodes*, the most commonly isolated organism in this study, is sensitive to the aromatic acetylenes and the thiophenes but not to the straight chain compounds **1**, **8** and **9**.

COMPARISON OF PHOTOTOXICITY OF SELECTED POLYACETYLENES TO *CRYPTOCOCCUS LAURENTII*

Two-fold serial dilutions of 5 µg/mL and 10 µg/mL of compounds **4**,**5**,**8** and **21** were added to approximately 1.69 X 10^4 cells/mL of *C. laurentii* in MYP. Control plates were kept in the dark and test plates were irradiated for 5,10 and 20 minutes. The optical density (O.D.) at 492 nm of each sample was read before irradiation (T_0) and at T_{20}h, T_{24}h, T_{40}h and T_{48}h later, and the differences expressed as a percentage of control O.D. (Tables XLII and XLIII). Percent survival of cells, 24 hours after exposure to varying concentrations of compounds was plotted against the time of UV-A irradiation (Figures 14 to 17). The synergistic action of all test compounds and UV-A irradiation on the viability of *C. laurentii* is demonstrated in these graphs.

The Minimum Inhibitory Concentration (MIC), causing complete growth inhibition, was 0.078 µg/mL for α-terthienyl after 10 minutes exposure to UV-A. No other compound was as lethal. Alpha-terthienyl also had a dark effect and killed some 20-30% of cells in the control plates at concentrations between 5 µg/mL and 0.039 µg/mL. None of compounds **4**, **5** and **8** (Figures 15 to 17) was completely lethal to *C. laurentii* even at 10 µg/mL. Higher concentrations were not used for two reasons: polyacetylenes precipitate in aqueous solutions

TABLE XLII. EFFECTS OF CHANGES IN POLYACETYLENE CONCENTRATION AND LENGTH OF UV EXPOSURE ON PERCENT SURVIVAL OF *Cr. laurentii**

Conc. (μg/mL)	Time of Irradiation (mins)			
	T_0	T_5	T_{10}	T_{20}
Compound 4**				
0.00	100%	100%	100%	100%
1.25	100%	79%	62%	41%
5.00	83%	67%	59%	39%
10.00	47%	53%	33%	19%
Compound 5				
0.00	100%	100%	100%	100%
1.25	98%	69%	57%	83%
5.00	77%	61%	72%	62%
10.00	46%	44%	52%	59%
Compound 8				
0.00	100%	100%	100%	100%
5.00	100%	61%	96%	95%
10.00	75%	60%	68%	62%
Compound 21				
0.00	100%	100%	100%	100%
0.0195	81%	68%	53%	NT***
0.039	83%	17%	14%	NT
0.078	75%	22%	3%	NT
5.00	73%	17%	5 %	NT

* All sampled incubated for 24 hours

** See Table IV-5 for compound names

*** NT not tested

FIGURE 14. EFFECT OF α-TERTHIENYL (21) AND UV-A ON THE 24 HOUR SURVIVAL OF *CRYPTOCOCCUS LAURENTII*

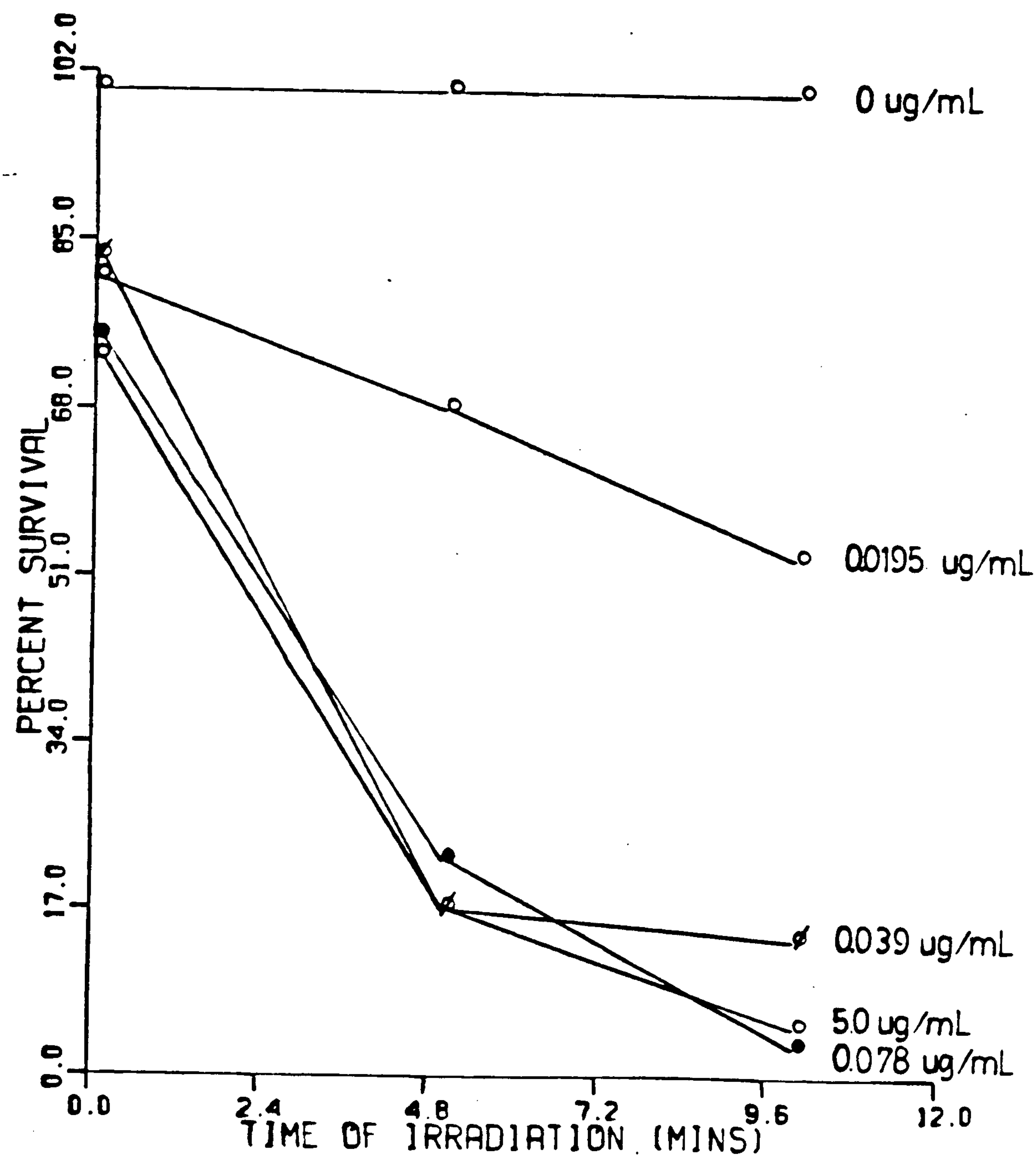

FIGURE 15. EFFECT OF PHENYLHEPTATRIYNE (3) AND UV-A ON THE 24HOUR SURVIVAL OF *CRYPTOCOCCUS LAURENTII*

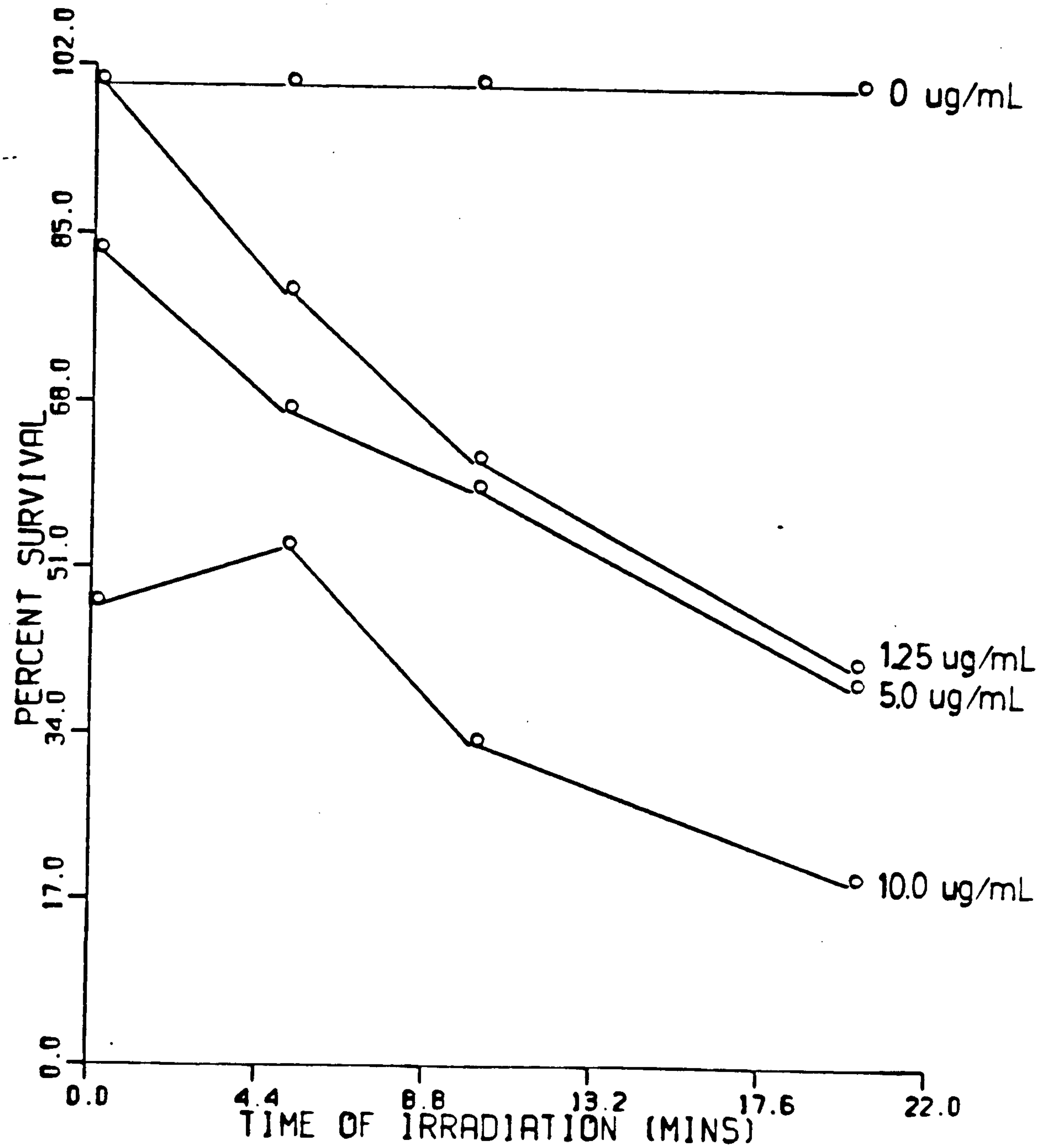

FIGURE 16. EFFECT OF PHENYLHEPTADIYNE-ENE (5) AND UV-A ON THE 24HOUR SURVIVAL OF *CRYPTOCOCCUS LAURENTII*

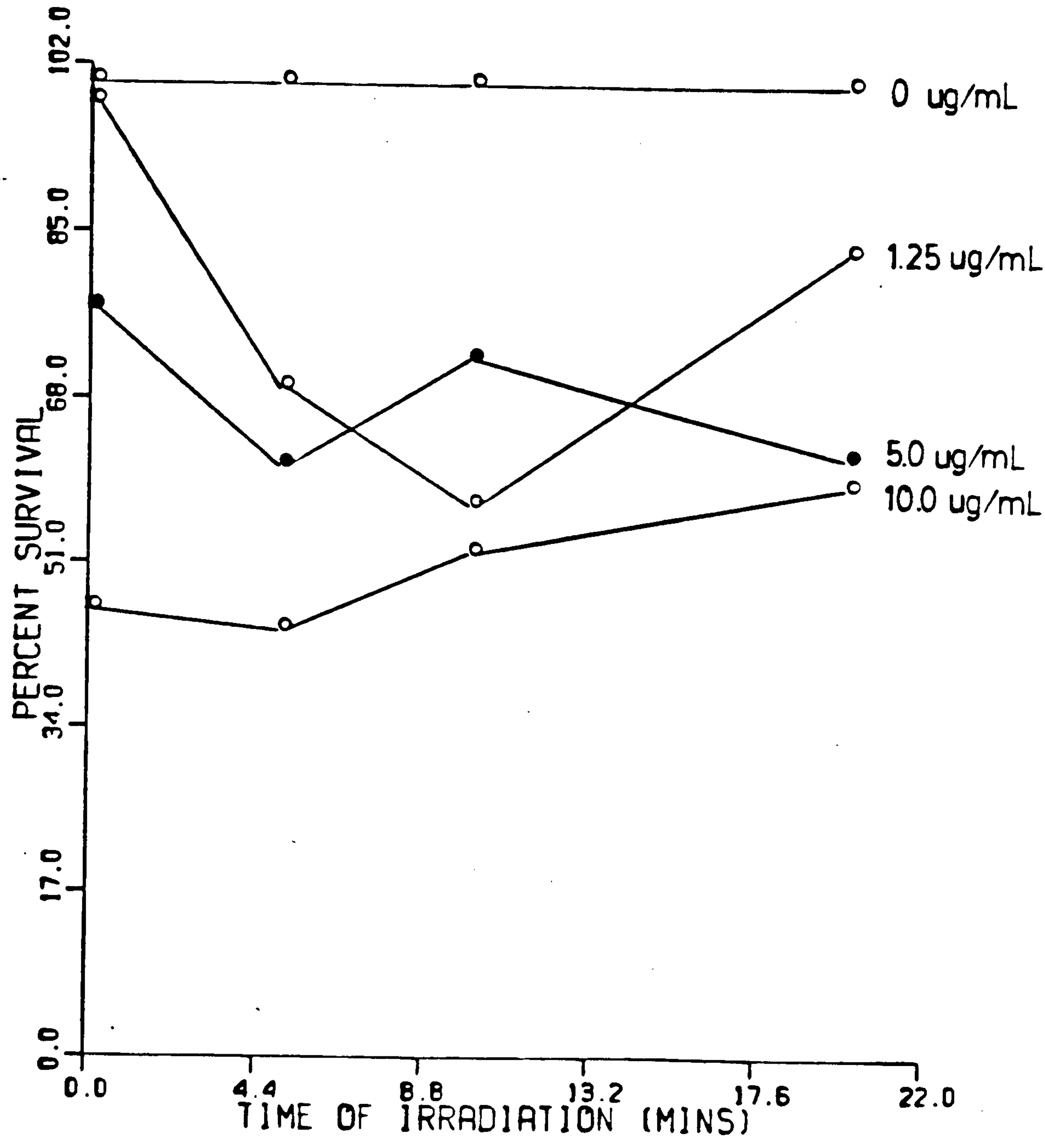

FIGURE 17. EFFECT OF HEPTADECA-TETRAENE-TRIYNE (8) AND UV-A ON THE 24HOUR SURVIVAL OF *CRYPTOCOCCUS LAURENTII*

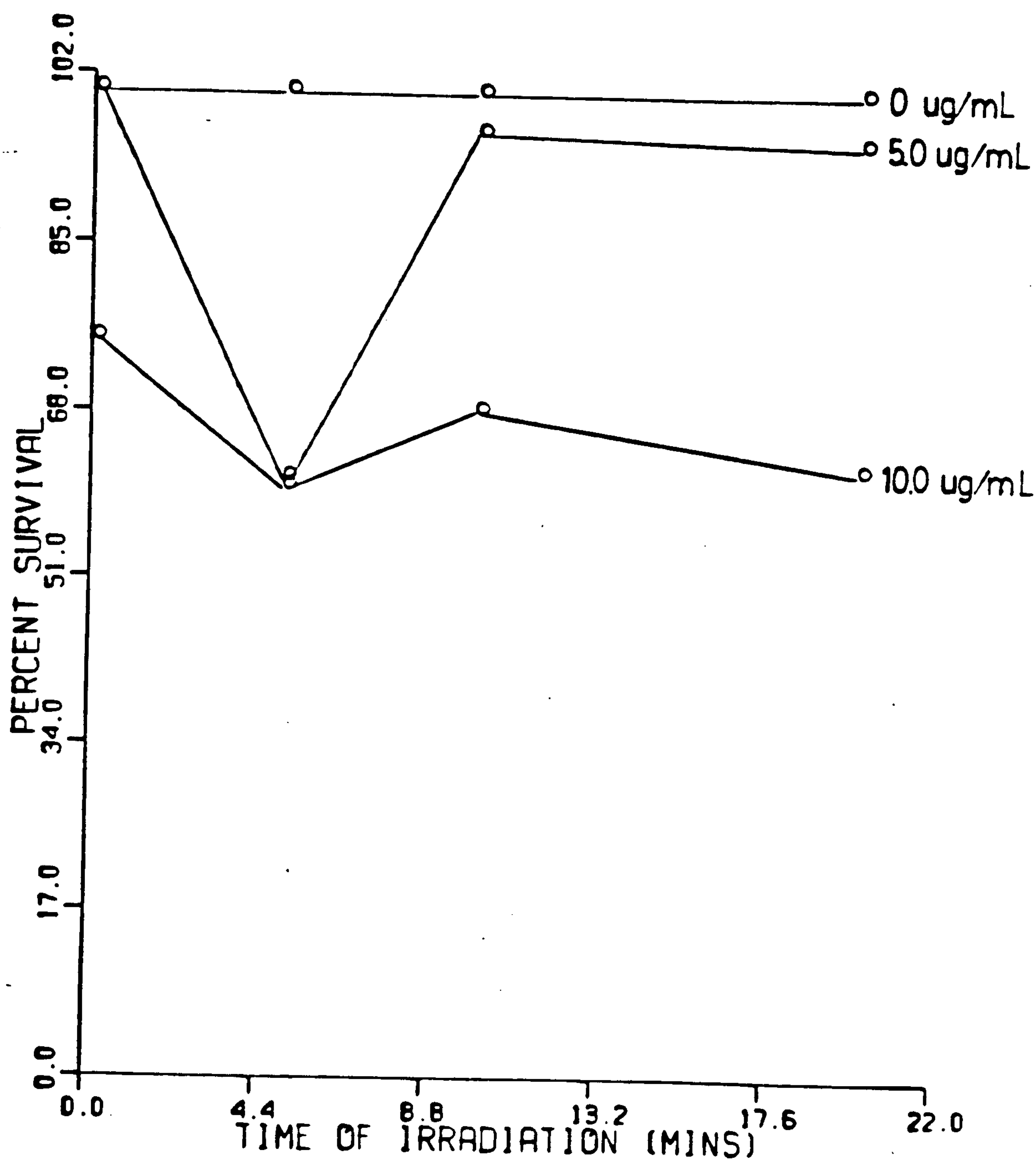

TABLE XLIII. SURVIVAL CURVES FOR *C. LAURENTII* EXPOSED TO POLYACETYLENES IN UV LIGHT*

Compounds**	Time of Incubation T_{20}	T_{24}	T_{40}
Control	100%	100%	100%
Compound 3	44%	59%	79%
Compound 5	50%	72%	88%
Compound 8	84%	96%	96%
Compound 21	9%	5%	4%

* 5 ug/mL exposed to 10 minutes UV light

** See table IV-5 for names of compounds at such concentrations and values of polyacetylenes in *Bidens* leaves do not exceed 10 μg/mL. Compounds **4**, **5** and **8** had an antibiotic effect at 10 μg/mL and compounds **4** and **5** also at 5 μg/mL. Irradiation of greater than 5 minutes duration seemed to cause breakdown of compounds **5** and **8**. This is not unexpected as polyacetylenes are known to be unstable in light and aqueous solutions (Towers, 1979). Figure 31 is a direct comparison of the phototoxic effects of the four test compounds against *Cr. laurentii*. At 5 μg/mL and after 10 minutes UV α-terthienyl is the most powerful photosensitizer, followed by PHT (**4**) phenyl-diyne-ene (**5**) and heptadeca-tetraene-triyne (**8**).

D. DISCUSSION

ISOLATION, IDENTIFICATION AND DISTRIBUTION OF PHYLLOPLANE MICROORGANISMS

Yeasts are defined by Kreger-van Rij (1984) as unicellular fungi reproducing by budding or fission, which may or may not be a stage in the life cycle of multicellular fungi. They are characterized by morphological and physiological criteria and are taxonomically diverse, including ascomycetes, basidiomycetes and imperfect fungi with both ascomycetous and basidiomycetous affinities.

In this study, 19 taxa of yeasts and yeast-like fungi were selectively isolated from 12 species of *Bidens* and 23 species of Hawaiian plants and their distribution recorded (Tables XXXII, XXXIV, XXXV, XXXVI). Isolation of filamentous fungi *per se* was limited by the nature of imminent phototoxicity assays which were originally developed for bacteria and yeasts (Fowlks, 1958; Daniels, 1965).

The data shows that, in Hawaii, just as in Indonesia (Ruinen, 1963) and temperate regions (Voznyakovskaya, 1962; Last and Deighton, 1965; Dickinson, 1976), healthy green leaves are inhabited by members of the Sporobolomycetaceae and Cryptococceae together with *Aureobasidium* and a few imperfect fungi such as *Cladosporium* and *Epicoccum* species. Using a leaf washing technique, Voznyakovskaya (1962) isolated a range of epiphytic microorganisms including *Sporobolomyces roseus*, *Rhodotorula rubra* (Demme) Lodder, *R.*

mucilaginosa, *R. aurantiaca* (Saito) Lodder, *Cryptococcus laurentii* and *C. albidus* from a diverse selection of hosts. Her survey served to stress the ubiquity of most leaf yeasts and also indicated the lack of host specificity among them. Phylloplane species also tend to be found more frequently on leaves than in soil, probably because they are well adapted to the microenvironment of the leaf. Many species are pigmented, which may be an adaptation enabling them to survive the high light intensity at the leaf surface (Last and Deighton, 1965; Last and Price, 1969; Pugh and Buckley, 1971; Ruscoe, 1971; Dickinson, 1976; McCauley and Waid, 1981).

Unlike those colonizing fruits and flowers, leaf yeasts are unable to ferment sugars (Last and Price, 1969), although some species have been shown to possess lipase activity which would enable them to become embedded into the wax layers of the cuticle. When *Cryptococcus laurentii* (Kuff.) Skinner and *Rhodotorula glutinis* (Fres.) Harrison were cultured on the stripped epidermis of *Aloe* sp. and the cuticle fragments of *Sansevieria* sp., the cuticles were seriously eroded within five days (Ruinen, 1966). Yeasts colonizing leaves above the cutin-reinforced anticlinal cell walls, may, as a result of their enzymic activity, detrimentally affect the intact cuticle and its functions. The phytopathogen, *Colletotrichum gloeosporiodes* enters the leaf by direct penetration without hyphal growth on the surface of the leaf (Blakeman, 1971; Marks *et al*.,1965) and is usually isolated from surface-sterilized leaves (Petrini *et al.*, 1982; Carroll and Carroll, 1978).

Despite the cosmopolitan distribution of phylloplane yeasts, the comparative study of Lamb and Brown (1971) suggests that the leaf itself may exert selective pressure which partially determines the nature of the resident phylloplane microflora on any given host. The specific nature of the pressure was not investigated or discussed. One of the objectives of this study was to determine whether the presence or absence of polyacetylenes in the leaves of Hawaiian *Bidens* exerts such an effect.

Numerous reports that polyacetylenes are photoactive against bacteria and fungi and other organisms (eg. Arnason *et al.*, 1980; Camm *et al.*, 1975; Chan *et al.*, 1975; DiCosmo *et al.*, 1982; Towers *et al.*, 1977; Wat *et al.*, 1977) have raised speculation about the putative biological functions of polyacetylenes in plants. Certainly the raison d'être of secondary compounds in general may never be fully clarified because a concerted, multidisciplinary, long-term approach is required for each class of chemicals, using appropriate source and test organisms (Janzen, 1979). Nevertheless, progress toward an answer may be made by small definitive steps. In this study, two specific questions were asked: does the presence or absence of polyacetylenes in Hawaiian *Bidens* leaves have any correlation with the distribution of selected phylloplane inhabitants and are these organisms sensitive to polyacetylenes?

In general, the nonfilamentous saprophytes isolated in this study occurred on both *Bidens* with leaf acetylenes and *Bidens* without acetylenes, as well as on most of the other plants sampled at any particular site (Tables XXXVI, XXXVII). It appears that the presence or absence of polyacetylenes within the leaves of *Bidens* is not correlated with the nature of the phylloplane yeasts and yeast-like fungi. Only *Colletotrichum gloeosporiodes* demonstrates discrete distribution. It was consistently absent from *Bidens* taxa which produce the C_{13} aromatic compounds **4**,**5** and/or **7** (Table XXXVII).

PHOTOSENSITIVITY OF PHYLLOPLANE MICRORGANISMS TO POLYACETYLENES

If polyacetylenes in leaves are photoactive against the microorganisms which dwell within or on the leaves, one might expect resident organisms to be unaffected by the compounds found in their host plants. Furthermore, phylloplane organisms from plants without leaf acetylenes would have no such resistance. Since most of the organisms were found on all plants sampled (Table XXXIV to XXXVI), one representative species from several hosts was checked for possible differential photosensitivity to acetylenes. *Cryptococcus laurentii* from *B. cosmoides* (compounds **1**,**4** and **5**), *B. hillebrandiana* ssp. *polycephala* (compounds **1**,**8**, and **9**), *B. sandvicensis* ssp. *confusa* (compound **5**), *B.*

sandvicensis ssp. *sandvicensis* (no leaf acetylenes) and *B. mauiensis* (no leaf acetylenes) were all tested for photosensitivity to the polyacetylenes listed in Tables XXXIII. No differences were detected (Table XL), which implies that the presence or absence of leaf acetylenes bears no relationship to the responses of *C. laurentii* to polyacetylenes.

Cryptococcus species, notably *C. laurentii*, have been isolated from northern and southern temperate regions, from the tropics and even from the Antarctic (diMenna, 1960; Last and Deighton, 1965), and are the only non-pigmented fungi found in this study. The incidence of pigmentation among phylloplane fungi and bacteria is high (70% among bacteria) (Ruinen, 1961; 1963a; Last and Deighton, 1965) and is thought to be an adaptation in response to UV radiation on exposed leaf surfaces. Ruscoe (1971), in a detailed direct examination study of *Nothofagus* leaves, found that hyaline species generally showed a hypophyllous distribution. *Phragmites* leaves, which are displayed in a nearly vertical position, had similar populations on both surfaces (Apinis *et al*., 1972). Although the significance of radiation as a determining factor may be debated, pigmentation itself may affect the degree of photosensitivity of organisms to specific acetylenes. Compounds 8 and 9 were found to be very toxic to bacteria and to *S. cerevisiae* and *C. albicans* (Tables XXXVIII and XXXIX), none of which are pigmented. These C_{17} compounds did not kill any of the pigmented phylloplane fungi but were phototoxic against all *Cryptococcus* species tested (Tables XL, XLI). *Rhodotorula* spp., pigmented relatives of *Cryptococcus* was resistant to compound **8** and **9**. *Epicoccum purpurescens*, *A. pullulans*, *S. roseus* and *R. pallida*, all pigmented species, seem to be resistant to most acetylenes occurring in *Bidens* (Table XLI). In addition, *S. roseus* is unaffected by 8-methoxypsoralen suggesting that it may have a metabolic mechanism for disabling this toxic furanocoumarin.

Dose response curves for compounds **4**,**5**, **8** and **21** were determined using *C. laurentii* as a representative phylloplane yeast because of its sensitivity to a wider range of polyacetylenes, which may or may not be because of its lack of pigmentation (Figures 14 to 17). The data in these graphs are generally in agreement with those obtained using the disc test (Tables XL, XLI), except that the Titertek method seems to be more sensitive because it revealed previously undetected antibiotic effects of these compounds.

The two methods are not directly comparable because one uses liquid suspensions of cells, unstable aqueous solutions of polyacetylenes of known concentrations and short irradiation times while the other uses solid medium, solvent-free acetylenes which diffuse in unknown quantities across agar and irradiation periods of up to two hours. These differences may account for discrepancies between data sets. For example, in Tables XL and XLI, compound **4** causes a wider zone of inhibition than compound **21** even though the data in Tables XLII and XLIII and Figures 14 and 15 indicate that the opposite should be true. This may be a reflection of the different modes of action of these two photosensitizers. The photodynamic action of α-terthienyl is oxygen-dependent whereas that of PHT is not (Arnason *et al*., 1980; Downum *et al*., 1982; McRae *et al*., 1985), and the two hour irradiation period in the disc test may favour the mechanism of action of PHT and enhance its toxic effect on the organisms. With the exception of *C. luteolus*, all *Sporobolomyces*, *Rhodotorula* and *Cryptococcus* yeasts in this study were more sensitive to PHT than α-terthienyl in the disc tests (Table XLI). It remains to be seen whether the opposite is true using the Titertek method.

Although the phototoxicity assays performed in this study provide an indication of the sensitivity of selected phylloplane organisms to polyacetylenes *in vitro*, such data cannot be unequivocally extrapolated to the situation *in vivo*. Toxicity *in vitro* does not prove toxicity *in vivo* although resistance *in vitro* implies resistance in any situation. All the fungi tested, with one exception (*Colletotrichum*), exhibit differential sensitivity to the test compounds. These responses are not related to the physical distribution of the organisms among *Bidens* (Tables XXXVII, XLI).

Unlike *B. alba* and *Coreopsis* species (Towers and Wat, 1978), in Hawaiian *Bidens* taxa there is no evidence for the presence of polyacetylenes in the leaf cuticle or within leaf surface structures such as trichomes. Resin canals exist but their minute diameters preclude sampling the contents for analysis. Whether acetylenes occur in resin canals, within cells or extracellularly, any contact between surface microorganisms and leaf acetylenes must occur within leaf tissue. Yeasts such as *Cryptococcus* and *Rhodotorula* spp., which degrade leaf cuticle to some extent (Ruinen, 1963b; 1966), may or may not encounter acetylenes, but species of *Colletotrichum*, as well as *Aureobasidium*, which penetrate leaf tissue and dwell within as endophytic pathogens (Blakeman, 1971), would be exposed to intra and extracellular constituents.

Colletotrichum gloeosporiodes was isolated from numerous species of Hawaiian *Bidens* and other plants sampled in this study. Its occurrence appears to be site related (Table XXXV, XXXVI). It was not found in *B. cosmoides*, *B. cervicata* or *B. sandvicensis* ssp. *confusa* and in only one other plant at the first five localities, in no other plant sampled at the third site. In six other localites where *Colletotrichum* was isolated from *Bidens*, two to four of the sympatric species were also hosts to the endophyte. Nevertheless, *C. gloeosporiodes* did not occur in *Bidens* which produce the C_{13} acetylenes (**1**,**4**,**5** and/or **7**). It was found to be very sensitive to **1** and **5** in the presence of UV radiation (Table XLI). Its sensitivity to α-terthienyl (Compound **21**) has been previously reported (di Cosmo *et al.*, 1982). It was not affected by compounds 8 and 9.

Although *in vitro* responses may not be a true reflection of the situation *in vivo*, this data suggests that the presence of polyacetylenes 1 and/or 5 in *B. cosmoides* (or *B. cervicata* or *B. sandvicenses* ssp. *confusa*) leaves precludes colonization of its tissues by *Colletotrichum gloeosporiodes*. The organism would presumably come into contact with polyacetylenes, which may be located extracellularly or within cells, as it invades the leaf and subsequently becomes inhibited by the photoactive compounds. There is also the possibility that some hitherto unknown phytoalexin(s) may be produced in response to fungal invasion. The selective photosensitivity may be caused by inherent morphological, physiological and/or biochemical characters specific to *C. gloeosporiodes* which causes it to react more strongly with some acetylenes than others and affecting its ability to grow within some plants.

Certainly this information is limited and cannot be interpreted as evidence for polyacetylene function *in vivo*. Nevertheless, it indicates that further research using phylloplane microorganisms on specific host plants may yield interesting information. Hawaiian *Bidens* not sampled in this study must be checked for *Colletotrichum*, especially, *B. campylotheca* ssp. *pentamera*, *B. torta* 17B and 17D and *B. valida*, all containing compounds 1,5 and/or 7 (Table V). Future investigations should focus on leaf-invading pathogens in order to determine whether there are other species excluded from *Bidens* leaves with specific polyacetylenes to which the organisms are sensitive, and whether there are pathogens which dwell within leaves which are resistant to the host acetylenes. Current bioassays for phototoxicity must be suitably refined and modified for filamentous fungi (Daniels, 1965; DiCosmo *et al.*, 1982). If such organisms can be found, a tentative argument for the case against the fortuity of polyacetylene phototoxicity to microorganisms may be made. As for the answer(s) to the central question of the putative role of polyacetylenes in nature, the complexity of the potential research problems to be surmounted cannot be overemphasized. These problems need to be carefully dissected into a methodical series of hypotheses which can be tested by experimentation. Parallel studies using different organisms and polyacetylene producing plants must be carried out in the laboratory as well as in the field. The present study has established that the phylloplane microflora/*Bidens* system is a useful model for further investigation.

E. CONCLUSION

Yeasts and yeast-like fungi were isolated from the leaves of Hawaiian *Bidens* and other plants and identified as members of the Sporobolomycetaceae, the Cryptococcaceae and the Fungi Imperfecti. All are common phylloplane inhabitants of worldwide occurrence. Although all of them are photosensitive to *Bidens* polyacetylenes, the distribution of nonfilamentous saprophytes among Hawaiian *Bidens* is not generally correlated with the presence or absence of polyacetylenes in the leaves.

It is significant that *Colletotrichum gloeosporiodes*, the only pathogenic species found in this study, is an endophyte which does not grow in *Bidens* leaves with C_{13} aromatic acetylenes to which it is extremely photosensitive *in vitro*. This suggests that further investigation of the relationship between leaf-invading pathogens, fungal and/or bacterial, and Hawaiian *Bidens* will be of great interest to those curious about the putative biological role of polyacetylenes.

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V. GENERAL CONCLUSION

In this dissertation several aspects of the biology and chemistry of polyacetylenes synthesized by the native Hawaiian species of *Bidens* were examined. The occurrence and distribution of acetylenes in these plants was consistent with the species concepts of Ganders and Nagata (1983) in their revision of the group, and with other evidence that the Hawaiian species are derived from a single ancestral lineage (Ganders and Nagata, 1984).

Some species have lost the ability to produce leaf acetylenes although *de novo* synthesis was clearly established in others. This trait is apparently dominant although acetylene inheritance seems complex and may be affected by the polyploid condition of the plants.

An endophytic fungus, *C. gloeosporiodes*, was discovered to be highly photosensitive to C_{13} aromatic acetylenes which occur in the leaves of *Bidens* species not inhabited by the organism. This seems to suggest that the presence of acetylenes in leaves may be a deterrent to colonization by certain fungal pathogens. This however, does not explain why some species no longer synthesize such leaf compounds.

Finally, an unusual aromatic thiophene was isolated from the roots of Hawaiian *Bidens* species. It has a unique combination of a phenyl ring and a thiophene ring bridged by a carbon-carbon triple bond. This compound may have interesting biological properties and merits future study.

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