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Title: EFFECTS OF TETRANYCHUS URTICAE KOCH FEEDING INJURY ON PHYSIOLOGICAL PROCESSES IN MENTHA PIPERITA L.

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Abstract approved: ____________________________  Gerald W. Krantz

Studies were conducted to examine the effects of feeding injury by the twospotted spider mite (Tetranychus urticae Koch) on physiological processes of its host plant, peppermint (Mentha piperita L.). Aspects of mite-induced host plant physiological stress that were studied included: 1) effects of injury on plant-water relations; 2) photosynthesis and leaf chlorophyll content; and 3) soluble leaf carbohydrates and starch. The effects of feeding injury are discussed in terms of underlying physiological mechanisms. In addition, new methods for sequential extraction and analysis of peppermint tissues are presented. Development of this methodology was necessary in order to satisfy the special requirements of these studies.

The most detrimental effect of feeding injury was damage to leaf epidermis and cuticle and the consequent alteration of
plant-water relations. Injured leaves were found to transpire more water at night than did uninjured leaves, resulting in symptoms of plant water stress the following day. These symptoms included reduced leaf water potential (psychrometrically determined) and stomatal closure. Levels of soluble peppermint leaf carbohydrates (mainly sucrose, raffinose, and stachyose) were higher on a per leaf basis in mite-injured leaves than in uninjured leaves. It is suggested that an osmotic adjustment mechanism, utilizing soluble carbohydrate as osmoticum, may be operating to maintain turgor in mite injury-induced water-stressed leaves. Stress-induced stomatal closure inhibited photosynthesis, presumably by restricting carbon dioxide exchange. In addition, mite feeding removed significant amounts of leaf chlorophyll, resulting in localized non-photosynthetic, necrotic patches.

An injury index based on the number of feeding adult female mites, duration of feeding, and leaf area is presented. This index was used throughout the studies to estimate the relative degree of injury of infested leaves.
EFFECTS OF TETRANYCHUS URTICAE KOCH FEEDING INJURY ON PHYSIOLOGICAL PROCESSES IN MENTHA PIPERITA L.

by

Jack Douglas DeAngelis

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TABLE OF CONTENTS

I. A REVIEW OF THE EFFECTS OF INJURY ON HOST PLANTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Biology of spider mites</td>
<td>2</td>
</tr>
<tr>
<td>Injury effects on host plant physiology</td>
<td>4</td>
</tr>
<tr>
<td>Effects on gas exchange</td>
<td>4</td>
</tr>
<tr>
<td>Additional effects on photosynthesis</td>
<td>6</td>
</tr>
<tr>
<td>Effects on plant growth and productivity</td>
<td>8</td>
</tr>
<tr>
<td>Conclusions</td>
<td>10</td>
</tr>
</tbody>
</table>

II. EFFECTS ON PLANT-WATER RELATIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>15</td>
</tr>
<tr>
<td>Introduction</td>
<td>16</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>21</td>
</tr>
<tr>
<td>Plant culture</td>
<td>21</td>
</tr>
<tr>
<td>Spider mite culture</td>
<td>22</td>
</tr>
<tr>
<td>Experimental design</td>
<td>23</td>
</tr>
<tr>
<td>Mite-days/cm² index</td>
<td>24</td>
</tr>
<tr>
<td>Leaf conductance</td>
<td>28</td>
</tr>
<tr>
<td>Osmotic potential</td>
<td>28</td>
</tr>
<tr>
<td>Leaf area and fresh weight</td>
<td>30</td>
</tr>
<tr>
<td>Results</td>
<td>31</td>
</tr>
<tr>
<td>Discussion</td>
<td>49</td>
</tr>
</tbody>
</table>
### III. SEQUENTIAL EXTRACTION AND ANALYSIS OF PEPPERMINT TISSUE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>56</td>
</tr>
<tr>
<td>Introduction</td>
<td>57</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>59</td>
</tr>
<tr>
<td>Plant culture</td>
<td>59</td>
</tr>
<tr>
<td>General extraction sequence</td>
<td>59</td>
</tr>
<tr>
<td>Column clean-up</td>
<td>62</td>
</tr>
<tr>
<td>Pigment and starch extraction</td>
<td>63</td>
</tr>
<tr>
<td>Analytical methods</td>
<td>64</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>68</td>
</tr>
</tbody>
</table>

### IV. EFFECTS ON PHOTOSYNTHESIS AND LEAF CHLOROPHYLL CONTENT

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>78</td>
</tr>
<tr>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>82</td>
</tr>
<tr>
<td>Experimental design</td>
<td>82</td>
</tr>
<tr>
<td>Leaf conductance</td>
<td>85</td>
</tr>
<tr>
<td>14-CO₂ Photosynthesis</td>
<td>85</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>90</td>
</tr>
</tbody>
</table>

### V. EFFECTS ON LEAF CARBOHYDRATES AND OSMOTIC ADJUSTMENT IN INJURED LEAVES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>103</td>
</tr>
<tr>
<td>Introduction</td>
<td>104</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>106</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>110</td>
</tr>
</tbody>
</table>

**BIBLIOGRAPHY** 119
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Method of calculating the mite-days/cm² injury index</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf conductance, measured during the day (EXP. II)</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf conductance, measured during the night (EXP. II)</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>Effect of <em>T. urticae</em> feeding injury on midday peppermint leaf osmotic potential (EXP. II)</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf weight (EXP. II)</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf weight (EXP. I)</td>
<td>43</td>
</tr>
<tr>
<td>7</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf area (EXP. II)</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>Effect of <em>T. urticae</em> feeding injury on specific weight (grams/cm²) of peppermint leaves (EXP. II)</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>Flow diagram of sequential extraction scheme</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>GC chromatogram of pentane fraction</td>
<td>67</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>Time course on <em>in situ</em> leaf starch hydrolysis</td>
<td>72</td>
</tr>
<tr>
<td>12</td>
<td>Soluble carbohydrates of peppermint by tissue type</td>
<td>73</td>
</tr>
<tr>
<td>13</td>
<td>Method of calculating the mite-days /cm² injury index</td>
<td>84</td>
</tr>
<tr>
<td>14</td>
<td>System for filling field tank with labelled air</td>
<td>88</td>
</tr>
<tr>
<td>15</td>
<td>Effect of <em>T. urticae</em> feeding injury on 14-CO₂ assimilation in peppermint</td>
<td>92</td>
</tr>
<tr>
<td>16</td>
<td>Effect of <em>T. urticae</em> feeding injury on chlorophyll content of peppermint leaves (EXP. I)</td>
<td>94</td>
</tr>
<tr>
<td>17</td>
<td>Effect of <em>T. urticae</em> feeding injury on chlorophyll content of peppermint leaves (EXP. II)</td>
<td>96</td>
</tr>
<tr>
<td>18</td>
<td>Relationship between photosynthesis and leaf conductance</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>Method of calculating the mite-days /cm² injury index</td>
<td>109</td>
</tr>
<tr>
<td>20</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf soluble carbohydrate (EXP. I)</td>
<td>112</td>
</tr>
<tr>
<td>21</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf soluble carbohydrate (EXP. II)</td>
<td>114</td>
</tr>
<tr>
<td>22</td>
<td>Effect of <em>T. urticae</em> feeding injury on peppermint leaf starch content</td>
<td>117</td>
</tr>
<tr>
<td>Table</td>
<td>Solubility of starch in aqueous ethanol</td>
<td>PAGE</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>69</td>
</tr>
</tbody>
</table>
Effects of *Tetranychus urticae* Koch Feeding Injury on Physiological Processes in *Mentha piperita* L.

CHAPTER I

A Review of the Effects of Injury on Host Plants
INTRODUCTION

Injury to crop and ornamental plants by the feeding activities of spider mites (Acari: Tetranychidae) causes considerable economic loss world-wide. Golik (1975) estimated that spider mites are responsible for annual losses amounting to 5% of the world's agricultural production. Severe infestation sometimes results in complete loss of the planting in annual cropping systems, and long-term loss of productivity in perennial systems. Despite these losses, and at a time when an increase in unit land area agricultural productivity is so vitally important, little information is available concerning the physiological mechanisms underlying spider mite injury to host plants. This review will summarize what is known about the effects of spider mite feeding injury on host plants, and help to define areas of investigation that may require attention.

BIOLOGY OF SPIDER MITES

The literature on spider mite biology is extensive (Jeppson et al. 1975), but comparatively few studies have addressed physiological aspects of spider mite/host plant interactions. As such, no attempt will be made to cover
general aspects of spider mite biology here, and emphasis will be on only those aspects pertinent to discussion of physiological interactions.

Tetranychids and their relatives have evolved specialized feeding structures that predispose them to feed on tissues of higher vascular plants (Krantz and Lindquist 1979). Long (100-150μM, partially fused cheliceral stylets are used to pierce living host plant tissues. The cheliceral bases are fused into a retractable stylophore which provides the motivating force for the stylets (Krantz and Lindquist op. cit.). Summers and Stocking (1972) observed that usually not more than one stylet puncture wound per epidermal cell was evident even in heavily mite-damaged almond leaves, but that a single puncture could represent injury to as many as 10 underlying palisade mesophyll cells (upper leaf surface feeding). Hislop and Jeppson (1976) noted that each insertion of the stylets is followed by repeated probing within the leaf, probably resulting in disruption of many subdermal cells. The manner in which cellular sap is transported from the leaf interior to the surface where it can be sucked up is still unclear. Hislop and Jeppson (op. cit.) reported that the inner or medial surface of each stylet is concave and, when appressed to its opposite, forms a channel through which sap may flow. It is unlikely that the pharyngeal pump is involved in sap
transport, however, because stylets and pharyngeal pump are separated.

SPIDER MITE FEEDING INJURY EFFECTS ON HOST PLANT PHYSIOLOGY

Effects on Gas Exchange. Higher land plants have evolved an extremely complex regulatory apparatus for controlling gas exchange with the atmosphere. Regulation of the flux of gases is needed to balance the opposing requirements of intensive gas exchange accompanying photosynthesis, and prevention of excessive water loss through transpiration. The stomatal apparatus (stomatal pore, guard cells, and subsidiary cells when present) and an epidermis covered with a cuticle of low permeability for water vapor and carbon dioxide, serve as gas exchange regulatory systems in higher plants (Raschke 1975). Effects of tetranychid feeding injury on gas exchange should therefore concern either effects on the protective epidermis and cuticle, or effects on stomatal function (including the substomatal diffusive resistance network), or both. Because of technical limitations, it has been difficult to distinguish between these two alternative mechanisms of injury.

Relatively few studies have examined the detrimental effects of mite feeding damage on gas exchange in host plants. Early studies concerning the effects of spider
mites on host plants dealt mainly with immediate and long-term effects on photosynthesis and transpiration, the two principle gas exchange processes, but did not address the underlying mechanisms of injury (Boulanger 1958, Wedding et al. 1958, Atanasov 1971, Kolodziej et al. 1974, Golik 1975, Hall and Ferree 1975). In general, it was found that injured leaves exhibited lower rates of both photosynthesis and transpiration than did uninjured leaves. Some investigators (Wedding et al. op. cit., Atanasov op. cit., Golik op. cit.) however, found that transpiration initially increased for a short time in injured leaves before the decline took place.

As was noted above, control of gas exchange in leaves depends on the integrity of the epidermal and cuticular layers, and on the substomatal diffusive resistance network. Two studies (Summers and Stocking 1972, Tanigoshi and Davis 1978) have demonstrated that considerable subdermal cellular disruption occurs at each spider mite feeding site. It is probable that collapse of mesophyll cells following stylet penetration and removal of cell sap would increase airspace volume within the leaf and thus have a direct effect on intercellular diffusive resistance of gases. Tanigoshi and Davis (op. cit.) also observed that penetrated epidermal cells were collapsed, suggesting direct effects on the epidermis and cuticle may be involved as well. Sances et
al. (1979a) suggested that depth of injury is dependent on feeding intensity, with more severely injured leaves exhibiting cell disruption at deeper levels. They also suggested that internal damage may affect the functioning of stomates through effects on tissue moisture status. Sances et al. (1979a) provided the first direct evidence of injury effects on stomatal function. Stomates of injured portions of strawberry leaves were found to exhibit a higher degree of closure than did stomates of uninjured regions. This closure would have had the effect of reducing gas exchange, provided that the epidermis and cuticle remained unaffected. In a second study (Sances et al. 1979b), both photosynthesis and transpiration rates were observed to decrease with increasing mite injury. Declines in the rates of both processes were attributed to increasing leaf resistance to gas diffusion (leaf resistance being the sum of stomatal and mesophyll resistances, by their definition). According to the interpretation of Sances et al. (1979b), leaf resistance increased primarily because of cellular damage to the mesophyll and increased mesophyll resistance.

Additional Effects on Photosynthesis. In addition to the effects on carbon dioxide exchange in injured leaves discussed above, mite injury causes other detrimental changes associated with the photosynthetic process.
Leaf chlorophyll content was lower in mite-injured leaves compared to uninjured leaves (Atanasov 1971, Kolodziej et al. 1974, Summers and Stocking 1972, Sances et al. 1979a). However, Sances et al. (1979b) was unable to demonstrate chlorosis in injured leaves when pigments were quantified spectrophotometrically after extraction in acetone. Spider mite feeding generally results in localized chlorotic areas called "stippling". As injury becomes more extensive, these areas coalesce into large chlorotic patches. Removal of photosynthetic mesophyll cells and their contents of chlorophyll, probably accounts for the reported diminution in leaf chlorophyll.

Summers and Stocking (1972) found almond leaves damaged by *Bryobia rubrioculus* to be significantly smaller than uninjured leaves, tending to be broad relative to their length, and somewhat altered in form. They suggested that feeding near the growing tip may retard axial growth, resulting in shorter leaves. Leaf area is directly related to photosynthesis in terms of the surface area over which the photosynthetic processes can operate. Leaf enlargement (expansion) is highly sensitive to leaf water status and even mild water stress can greatly reduce or halt expansive growth (Boyer 1976).

Ultrastructural changes in chloroplasts of cells adjacent to feeding sites of *Tetranychus mcdanieli* on apple
leaves have been reported (Tanigoshi and Davis 1978). Marked swelling of chloroplasts and morphological alteration of stromal and granal thylakoid membranes were symptomatic of cells adjacent to these sites. Tanigoshi and Davis (op. cit.) suggested these changes were reminiscent of those occurring during natural senescence, pathogenic infection, cold and chemical injury, and mineral deficiencies. It seems likely that these changes in the lamellar membrane systems of chloroplasts would have profound effects on the photosynthetic process as both electron transport and photophosphorylation are membrane-associated phenomena.

Effects on Plant Growth and Productivity. Effects of spider mite feeding injury on transpiration and photosynthesis are manifested as detrimental effects on vegetative growth, and ultimately fruit production. Because the leaves on which spider mites feed are the primary producers of organic nutrients in plants, mite injury effects can ultimately be transmitted to the whole plant. Effects on leaf water status resulting from alterations in the transpiration stream may also affect the entire plant. Reduction in trunk diameter (Chapman et al. 1952), stem and root biomass (Briggs and Avery 1968), and flower bud formation (Chapman et al. op. cit., Lienk et al. 1956, Golik 1975) in spider mite-damaged fruit trees have been reported. Fruit yield
and quality may also be negatively affected by spider mite
injury (Lienk et al. op. cit., Golik op. cit.).

A particularly interesting aspect of spider mite/host
plant interactions is the delayed response of some
perennials to spider mite injury. Barnes and Andrews (1978)
found that almond trees infested by *Tetranychus* spp. and
*Panonychus citri* showed no reduction in yield during the
year in which the infestation occurred, but that in the year
following the infestation, yields were reduced 13-19%. A
similar situation was reported by Klopfenstein and
Holdsworth (1978) for apple trees infested by *Panonychus
ulmi*.

Several studies have demonstrated that growth may be
affected in spider mite-injured plants because of
alterations in the normal balance of growth regulator
substances (Avery and Briggs 1968, Avery and Lacey 1968,
Storms 1971). Avery and Briggs (op. cit.) showed that when
*Panonychus ulmi*, that had been previously labelled with
14-carbon, were allowed to feed on fruit tree leaves, label
appeared at uninfested growing points, indicating intraplant
transport of the tracer. Avery and Lacey (op. cit.)
demonstrated differences in growth regulator content of plum
shoots after mite infestation. Storms (1971) found saliva
of *Tetranychus urticae* was transported to growing regions of
bean plants. Enhanced transport of phosphates, as well as
increased levels of gibberellin, were also found in infested plants (Stormes op. cit.).

CONCLUSIONS

One conclusion that may be drawn from a review of the literature on the effects of spider mite feeding injury on host plant physiology is that more detailed research is needed on the mechanisms underlying injury. It is evident that three general types of leaf injury result from spider mite feeding; 1) simple mechanical damage to the protective leaf epidermis and cuticle caused by stylet penetration, 2) removal of photosynthetic mesophyll cells, and 3) a less obvious, and experimentally more difficult to confirm, influence on growth regulator substances. In addition, injection of toxic materials, such as digestive enzymes, may also be involved in the general plant responses to spider mite attack.

The work of Duniway and Durbin (1979a,1979b), on the effects of rust infection (Uromyces phaseoli) on water relations in bean, may serve as an example of the kind of detailed study concerning mechanisms of injury that could be applied to research on spider mite/host plant interactions. Rust sporulation on leaf surfaces causes cuticular and
epidermal damage similar to that caused by spider mite injury. In both cases the damage is mainly mechanical, leading to changes in transpiration of the affected leaves, and making them more susceptible to drought and leaf wilt (Duniway and Durbin 1979b). McCoy and Albrigo (1975) also applied a more detailed type of approach in studies investigating the mechanisms of feeding injury by the eriophyid mite Phyllocoptruta oleivora on orange. Eriophyid mites, because of the very short length of their stylets (ca. 10μm), feed only on epidermal cells. McCoy and Albrigo found that mite injury caused a significant increase in ethylene emission from injured cells, as well as cytological changes associated with wound periderm formation. Eriophyid mite injury seldom results in immediate cell death, in contrast to spider mite injury, and hence the tissues are subject to long-term morphological changes resulting from plant injury responses (McCoy and Albrigo op. cit.).

Detrimental effects on gas exchange processes in leaves are probably the most serious consequences of tetranychid feeding injury. Gas exchange is normally closely regulated by complex active and passive feedback mechanisms that have evolved to coordinate the demand for carbon dioxide, with control of water loss (Raschke 1975). A series of barriers, or resistances to gas diffusion are the points at which control is exerted in the plant's gas exchange regulatory
system. The magnitude of the resistance to gas diffusion of most of these barriers is invariable, at least over short time spans; the magnitude of the stomatal resistance component, however, is diurnally variable. The resistance components which normally cannot be rapidly varied include the epidermis and associated cuticle, the intercellular air space pathway resistance, and the mesophyll cell wall liquid-phase pathway resistance. Stomatal movements provide a variable resistance component and thus serve to regulate gas fluxes between leaf and air diurnally (Nobel 1974). Spider mite feeding causes collapse of epidermal cells and disruption of overlying cuticle, probably reducing the effective magnitude of this outermost barrier to gas diffusion. The resulting uncontrolled water loss may account for the observed water deficit effects in spider mite-injured leaves. Destruction of mesophyll cells would affect the intercellular air space pathway resistance by altering spacial relationships between cells, however the ultimate effects of this on plant water relations and photosynthesis are not easily predicted.

The loss in plant productivity that is symptomatic of spider mite injury may be viewed in terms of a shift in resource allocation from reproductive growth (our measure of productivity), to maintenance of the vegetative plant body. The mechanisms of mite injury responsible for this shift are
not known, however, some generalities can be stated: 1) plant water stress, whether brought on by biotic or abiotic factors, results in stomatal closure that limits carbon dioxide flux into the leaf, and consequently reduces available photosynthate, 2) destruction of photosynthetic mesophyll cells by spider mite injury also reduces the available surface area for photosynthesis, 3) injury may hasten leaf senescence through general wound-reaction mechanisms, causing premature leaf drop, and 4) injection of growth regulator substances (plant hormones or analogs) may alter the distribution of available resources.
Effects of *Tetranychus urticae* Koch Feeding Injury on Physiological Processes in *Mentha piperita* L.

CHAPTER II

Effects on Plant-Water Relations
ABSTRACT

Studies were conducted to examine effects of feeding injury by the spider mite *Tetranychus urticae* Koch on the water relations of its host plant *Mentha piperita* L. The principle objective was to determine if observed water stress in injured leaves could be accounted for by simple mechanical damage to the leaf epidermis and cuticle. Leaf conductance to water vapor diffusion of uninjured and variously injured leaves was determined during the day and at night in order to assess the relative magnitudes of water vapor transport through the stomatal and cuticular pathways. In addition, several parameters relating to leaf water status were determined concurrently with daytime leaf conductance measurements. These included leaf osmotic potential, leaf fresh weight, and leaf area. Injured leaves exhibited lower leaf conductance to water vapor diffusion during the day and higher conductance at night. The differences were correlated with degree of injury as estimated by an injury index developed for this study. High leaf conductance at night resulted in leaf water stress during the day in mite-injured leaves as evidenced by more negative leaf osmotic potential and decreased leaf area, fresh weight and specific weight (leaf weight per unit area).
INTRODUCTION

The overall objective of this research has been to characterize host plant stress resulting from spider mite (Tetranychidae) feeding injury. To this end a number of physiological changes in peppermint (Mentha piperita L.), associated with feeding injury by the twospotted spider mite, Tetranychus urticae Koch, have been examined. Experiments presented here investigated effects of feeding injury on water relations of peppermint.

In previous studies concerning effects of spider mite injury on host plant physiology, the predominant and most obvious effects reported were those on water loss or transpiration. From one of the first studies of this kind, Wedding et al. (1958) reported initial increase in water loss from injured citrus leaves, followed by long-term reduction to levels approaching 80% of uninjured leaves. Cyclic patterns of water loss following mite injury have been shown by other workers for other plant species. Boulanger (1958) observed a similar but less pronounced pattern in apple leaves damaged by Panonychus ulmi, with the strongest tendency toward reduced transpiration with increasing severity of injury. Atanasov (1971) found that injured bean leaves transpired at three times the rate of
uninjured leaves, although the usual pattern of long-term reduction in transpiration was not reported. In studies reported by Golik (1975), severely mite-injured and uninjured apple leaves exhibited lower rates of water loss than did moderately injured leaves. Recent studies by Sances et al. (1979) showed, in general, a negative correlation between water loss and mite injury in strawberry.

Spider mites feed in a rather unusual way; they neither suck plant juices in the manner of aphids, nor do they chew host plant tissue. Paired and partially fused cheliceral stylets are inserted through epidermal cells and into underlying mesophyll cells, and act to puncture cells and disrupt the spongy and palisade cell layers (Jeppson et al. 1975). The manner in which the cellular fluid is transported from the interior of the leaf to the surface where it can be sucked up is unclear, although some type of capillary flow along the stylets may be involved. Cell wall material from disrupted mesophyll is not ingested and remains behind in the leaf. Destruction of underlying photosynthetic cells results in localized chlorotic areas referred to as "stippling". As feeding injury becomes more severe these chlorotic areas may coalesce, indicating considerable subdermal damage.
The principle question addressed in this study was: can the observed effects on transpiration in mite-injured leaves be accounted for by simple mechanical damage to leaf epidermis and cuticle? In other words, are spider mites opening new pathways for water vapor diffusion, and hence water loss, by disrupting the cuticle and epidermis through their feeding activities?

Transpirational water vapor flux per unit leaf area, symbolized \( J_W \) (g/cm\(^2\)/sec), from mesophyll cell walls to the turbulent air surrounding a leaf is described by the steady state relation: (Nobel 1974)

\[
J_W = \frac{\Delta C_W}{(R^l + R^B)} \quad \text{equation 1}
\]

where \( \Delta C_W = (C^m - C^a) \) = the difference in water vapor (w) concentrations between mesophyll cell wall pores (m) and turbulent air (a) surrounding the leaf (g/cm\(^3\)), and \( (R^l + R^B) \) = total diffusive resistance to water vapor transport encountered along the pathway of mesophyll cell wall pores to turbulent air (sec/cm).
Leaf resistance to water vapor diffusion ($R^l$) is the sum of two parallel components—stomatal resistance ($R^s$) and cuticular resistance ($R^c$). Stomatal resistance, in turn, is the sum of two series resistance components—intercellular air space and stomatal pore resistance. Stomatal movements exercise the greatest control over transpiration in healthy leaves through their effects on stomatal resistance, (Nobel 1974, Raschke 1975). Transpiration through the cuticle is usually low (high) because the waxy cuticle (epicuticular wax, Esau 1977) retards water vapor diffusion across this pathway.

By analogy to electrical circuit theory, the sum of parallel resistors is expressed as $\sum \frac{1}{R_i}$, where $R_i$ is the resistance of parallel resistor $i$. From this relationship, leaf resistance is calculated by: (Nobel op. cit.)

$$R^l = \frac{R^s \cdot R^c}{(R^s + R^c)}$$

equation 2

Boundary layer resistance, $R^b$, results from the still, laminar air layer adjacent to leaf surfaces and is in series with leaf resistance.
Leaf conductance to water vapor diffusion is calculated as the reciprocal of the resistance terms in equation 1:

$$L_W = \frac{1}{(R^t + R^b)}$$

where $L_W$ = leaf conductance (cm/sec)

In order to investigate effects of spider mite feeding injury on transpiration of peppermint leaves, leaf conductance was determined on healthy and variously injured leaves at night and during the day. This approach enabled segregation of the relative contributions of stomatal and cuticular pathways to diurnal water loss. In addition, several parameters of leaf water status were determined concurrently with daytime leaf conductance measurements to assess the effects on plant-water relations. Parameters included leaf osmotic potential (psychrometrically determined), leaf area, and leaf weight.

Significant effects on leaf conductance were found in spider mite-injured leaves. These effects were translated into symptoms of plant water stress during the day. The principle and most serious effect was a substantial increase in leaf conductance to water vapor diffusion at night, resulting in lowered leaf water status during the day.
METHODS AND MATERIALS

Two series of experiments were conducted. Both series were very similar in design, with only minor differences as noted below. The first experiments, designated as "EXP. I", were intended to supply preliminary data on which later experiments could be based. The majority of data and methods to be present below are concerned with the second series of experiments, "EXP. II".

PLANT CULTURE. Black Mitcham peppermint plants (Willamette Valley clone) were grown from field-collected rhizomes in beds of vermiculite. Beds were cut back at approximately three week intervals to maintain vigorous vegetative growth (Crane and Steward 1962). At the time of the second cutting when beds were ca. 45 days old, individual tips, consisting of the stem bearing the tuft of youngest leaves at the apex and one or two expanded leaves below the apex, were cut from the plants and planted in silica sand in rooting tubes. Both stock rhizome beds and cuttings in silica sand were watered with a modified Hoagland-Arnon nutrient solution (Hoagland and Arnon 1938, as modified by Crane and Steward op. cit.).

Ambient conditions inside the growth chamber were
controlled throughout the experiments and maintained as follows: 16 hr light; 23-25°C day/20-22°C night temperature regime; 40-60% relative humidity. Light was provided by a specially constructed bank of VHO Wide Spectrum Gro-Lux lamps, capable of high levels of light output (upto ca. 1000 microEinsteins/m²/s (μE/m²/s) at the level of the cuttings). Plants were grown under ca. 250 μE/m²/s. Quantum flux was measured with a LiCor Quantum/Radiometer/Photometer, model 185A (LiCor Inc., Lincoln, Nebraska), in the quantum mode. Plexiglas filters were fitted over the lamps to protect plants against UV radiation, to which peppermint is sensitive (unpublished data).

Cuttings were allowed to root under low light (<100 μE/m²/s) for two weeks before being transferred to the higher light conditions. During the rooting period, peppermint cuttings are highly susceptible to water stress caused by high light intensity or high temperature. Water stress during rooting can affect the morphology of unexpanded leaves, causing them to develop abnormally (unpublished data). Conditions that might have lead to water stress in cuttings were therefore avoided where ever possible.

SPIDER MITE CULTURE. Stock cultures of *Tetranychus urticae* were maintained on Henderson bush lima bean plants according
to the methods of Scriven and McMurtry (1971) and Gilstrap (1977). Subcultures were also maintained on peppermint because mites reared on beans for long periods of time did not readily accept peppermint as a host plant (unpublished data). All of the mites used in the experiments were taken from the peppermint culture.

EXPERIMENTAL DESIGN. Advantage was taken of the paired and opposite leaf arrangement of peppermint in setting up the sampling schemes. Third or fourth leaf pairs below the apex of three (EXP. I) or five (EXP. II) week old cuttings were used throughout the studies. One leaf of a pair was infested with virgin female *T. urticae* at rates of 2, 5, 10, or 20 mites per leaf while the opposite leaf remained uninfested to serve as an intra-plant control. Leaves were infested by hand-transferring quiescent female deutonymphs or newly molted adults to leaves. Mites were confined to infested leaves by a barrier of Tack Trap (Animal Repellents Inc., Griffin, GA.) encircling each petiole. This material proved to be an effective barrier to spider mite dispersal. Unmated females were used in order to take advantage of the fact that spider mites are arrhenotokous; consequently unmated females produce only male progeny (Jeppson et al. 1975). Immature males are smaller and probably feed less than do their female counterparts and hence contribute
little to any error arising from basing degree of injury on a count of adult females per leaf (see below).

Following the two week rooting period, 112 of the original 175 rooted cuttings (EXP. II) were selected for uniformity, and randomly assigned to rooting-tube trays (one tray for each of the sampling days). Four plants in each tray were infested at each of the prescribed infestation levels. Samples were taken at 3, 6, 9, 11, 13, 15, and 17 days after infestation.

For EXP. I, 180 of an original 250 rooted cuttings were selected as described above. Infestation of leaves was at rates of 0, 2, 5, 10, and 20 adult female *T. urticae* per treatment leaf. Samples were taken at 0, 2, 4, 6, 8, 10, 12, 14, and 16 days after infestation.

*MITE-DAYS/CM^2 INDEX*. There have been a number of indices used relating degree of host plant injury to level of spider mite infestation. The simplest has been relative ranking of mite densities to levels of low, medium, and high, and sometimes controlling populations with selective application of miticides (*Barnes and Andrews* 1978, *Klopfenstein and Holdworth* 1978). *Golik* (1975) used a ranking system of five levels, with each level defined by visual symptoms of leaf injury. Levels ranged from uninjured healthy leaves (level 1), to very heavily damaged and bronzed leaves (level 5).
Injury indices have been based more commonly on numbers of mites per leaf, usually measured as initial infestation in cases of artificially manipulated populations (Wedding et al. 1958, Atanasov 1971, Rasmy et al. 1974).

Kolodziej et al. (1974) introduced the idea of including leaf area in the measure of infestation level. They also restricted their counts of feeding mites to adult females. Mite-days was introduced to account for changes in the initial population of feeding mites through time (Hoyt et al. 1979). Mite-days was calculated as \([(initial\ count + final\ count)/2]\ multiplied by the number of days between sampling periods, inclusive. Hoyt et al. (op. cit.) gave no indication as to which stages or sex of mites were counted; presumably all mites were included in their samples. Sances et al. (1979a, 1979b) employed the index of Hoyt et al. (op. cit.) in studies on strawberry in which mites were confined to 2 cm² arenas on leaf undersides. The mite-days level was divided by the area of the arenas to arrive at a final index of mite-days/cm².

The index used in this study (Fig.1) was similar to that of Sances et al. (op. cit.) except that counts of initial and final population levels were used as estimates of mean population in the manner of Hoyt et al. (op. cit.), and the area in the denominator was the area of the uninfested leaf of the pair, computed as described below.
FIGURE 1. Method of calculating the mite-days/cm² injury index used in these studies. "COUNT" refers to the number of living adult female *T. urticae* at the beginning (initial infestation level) of the experiment (N=0) and at each sampling day (N).
Mite-Days $\frac{1}{cm^2} = \frac{\left(\text{COUNT}_0 + \text{COUNT}_N \div 2\right) \cdot N}{\text{AREA OF UNINFESTED LEAF (cm}^2\text{)}}$

where: $0$ & $N$ = DAYS AFTER INFESTATION

Figure 1
Only adult female mites were counted.

Only the mite-days index, calculated as mite density x duration of feeding (days), was used in EXP. I because leaf area measurements were not made.

LEAF CONDUCTANCE. Leaf conductivity was measured using the LiCor LI-65 Autoporometer (LiCor Inc., Lincoln, Nebraska). Calibration and temperature correction was done according to the methods of Berkowitz (1980). Leaf conductance was determined on lower leaf surfaces at two times for each leaf: once at 1 hr into the dark period, and again at 8 hr into the light period (15 hr later). Each determination was the mean of two measurements per leaf except that all leaves having leaf conductance values >67 sec/cm (autoporometer transient times >60 sec), i.e. only certain leaves sampled during the dark period, were recorded as 80 sec/cm. In nearly every case these leaves had values in excess of 80 sec/cm and therefore the readings were more a function of instrument drift then actual leaf resistance.

OSMOTIC POTENTIAL. The chemical potential of water (= water potential, \( \Psi \)) in a plant cell is governed by three interacting components: hydrostatic or turgor potential (\( \Psi_p \)), matric potential (\( \Psi_T \)), and osmotic potential (\( \Psi_i \)). The relationship between these components of water potential
is expressed as:

\[ \psi = \psi_p + \psi_\pi + \psi_T \]

The distribution of the components of water potential of a plant cell varies with location as shown by Turner (1981). The contribution of the matric component in the vacuole to the water potential of the cell is very low or absent (Turner op. cit.), and as such this term is often regarded as negligible and included in \( \psi_p \) or \( \psi_\pi \). The expression for water potential then becomes:

\[ \psi = \psi_p + \psi_\pi \]

Osmotic potential of infested and uninfested leaves was measured psychrometrically using the Wescor C-52 sample chamber and HR-33T Microvoltmeter (Wescor Inc., Logan, Utah) in the psychrometric mode.

Leaf disc samples were killed by immersion in liquid nitrogen before placing them in the sample chamber, which allowed determination of the osmotic component of leaf water potential (turgor pressure falls to zero on killing the tissue in liquid nitrogen (Brown 1972)). Microvolt output from the microvoltmeter was recorded on a stripchart recorder (Omega Engineering, Inc., Stamford, Conn., model 555). Each determination was the mean of two measurements per sample. Equilibration times of 15-20 minutes were found
to be adequate for most samples. Calibration to NaCl standards was performed daily and was consistent throughout the experiment.

LEAF AREA AND FRESH WEIGHT. Leaf area was estimated using a linear regression model determined from a set of similar leaves. The product of the measurements of leaf length and width at the widest point was found to be closely correlated with area as determined with a LiCor Area Meter model LI-3000 (LiCor Inc., Lincoln, Nebraska), according to the following regression model:

\[
\text{Leaf Area (cm}^2\text{)} = 0.44 + 0.703 (\text{length} \times \text{width (cm)})
\]

\[r^2 = 0.97\]

Leaves were weighed on an analytical balance (Mettler model H10) immediately after harvesting.

All statistical analyses were done on the Cyber computer system at Oregon State University, using the SIPS interactive statistical package.
RESULTS

The results of these studies can be summarized as follows:

1) Feeding damage by *Tetranychus urticae* caused marked changes in rates of water loss from leaves of *Mentha piperita*. When measured during the day, when stomates are normally open, injured leaves transpired at lower rates than did uninjured leaves. The magnitude of the difference was correlated with degree of injury as estimated by the mite-days index. When measured during the night, a period when stomates are normally closed, injured leaves showed higher rates of water loss, and the difference was again correlated with the mite-days index.

2) The osmotic potential of injured leaves was found to be lower (more negative) than that of uninjured leaves during the day, indicating that injured leaves may be experiencing water stress due to feeding injury.

3) The fresh weight of injured leaves was lower than that of uninjured leaves and the difference was linearly correlated with degree of injury.

4) A minor but statistically significant effect of mite-injury on leaf area was found. The magnitude of the
effect was observed to be dependent on leaf age.

5) Specific leaf weight (fresh weight per unit area) was not significantly affected in injured leaves until high injury levels were reached.

Leaf conductance to water vapor diffusion, measured during the day, was found to decrease with increasing mite injury (Fig. 2). At the highest injury levels (15-20 mite-days/cm²) injured leaves conducted only ca. 50-60% of the water vapor that uninjured leaves did. The response of leaf conductance was correlated with degree of injury as estimated by the mite-days cm⁻² index ($r^2 = 0.27; P < 0.001$). Most of the variability inherent in these data can probably be attributed to normal variation in stomatal opening under the range of ambient lighting conditions of the experiment. The response of leaf conductance was linear, decreasing by ca. 10% with each 5 mite-days increment of injury (Fig. 2), indicating a rapid and sustained stomatal response to mite injury. This response would have the effect of lowering the rate of water loss from injured leaves during the day.

The response of leaf conductance to feeding injury at night, when stomates are normally closed, was the reverse of that found during the day, and much more pronounced (Fig. 3). Leaf conductance increased at a linear rate of 14.5 percent per increment of injury. At the highest injury level injured leaves exhibited leaf conductance values of
FIGURE 2. Effects of *T. urticae* feeding injury on peppermint leaf conductance, measured during the day (EXP. II). The Y-axis is the ratio of leaf conductance of injured to uninjured leaves, expressed as a percent \(((\text{injured}/\text{uninjured})\times100)\). Each point represents the mean of 4 replicates. \(100.3 - 2.3X, r^2 = 0.27, P<.001, n=112\).
Figure 2
FIGURE 3. Effects of *T. urticae* feeding injury on peppermint leaf conductance, measured during the night (EXP. II). The Y-axis is the ratio of leaf conductance of injured to uninjured leaves, expressed as a percent ((injured/uninjured) × 100. Each point represents the mean of 4 replicates. 112.6 + 14.46X, $r^2 = 0.54$, $P < .001$, n=112.
Figure 3

Leaf Conductance (% of uninjured leaves) vs. Mite-Days cm$^{-2}$

NIGHT L$_{wv}$

uninjured leaves
ca. 400% of uninjured leaves. The response was correlated with degree of injury ($r^2 = 0.54; P<.001$). As with the response of leaf conductance during the day, leaf conductance measured at night responded rapidly to mite injury, exhibiting little or no lag period.

Osmotic potential of injured leaves was found to be lower at midday than that of uninjured leaves (Fig. 4). The relationship of osmotic potential and injury was linear, decreasing at a rate of ca. 0.23 bars per increment of injury, and was correlated with injury ($r^2 = 0.46; P<.001$).

Figures 5 and 6 show the change in leaf fresh weight, expressed as percent of uninjured leaves, as a function of mite injury. Figure 5 shows the data from Exp. II and Fig. 6 the data from Exp. I. If suitable corrections are made for the difference between the scales of the X-axes (Mite-Days versus Mite-Days/cm²), the relationship between fresh weight and injury was nearly identical in both studies, leaf weight decreasing at a similar rate in both cases.

Leaf area measurements were not made during Exp. I. It was observed however that injured leaves tended to be smaller than uninjured leaves, and hence provisions were made for obtaining these data during EXP. II (Fig. 7). The effect of injury on leaf area, measured during EXP. II, was not as pronounced as that observed during EXP. I. Figure 7
FIGURE 4. Effect of *T. urticae* feeding injury on midday peppermint leaf osmotic potential (EXP. II). The Y-axis is the difference in osmotic potential between uninjured and injured leaves (uninjured – injured). Each point represents the mean of two determinations per sample. $0.198 - 0.23X$, $r^2 = 0.46$, $P<.001$, $n=26$. Mean leaf osmotic potential for uninjured leaves = -8.88 bars (SD = 1.35).
Figure 4
FIGURE 5. Effect of *T. urticae* feeding injury on peppermint leaf weight (EXP. II.). The Y-axis is the ratio of leaf weights of injured to uninjured leaves, expressed as a percent \(((\text{injured/uninjured}) \times 100)\). Each point is the mean of 4 replicates. $100.9 - 0.94X$, $r^2 = 0.24$, $P < .001$, $n=112$. The mean leaf weight of uninjured leaves = 0.1851 grams (SD 0.06).
Figure 5

LEAF FRESH WEIGHT

Weight (% of uninjured leaves)

Mite-Days cm$^{-2}$

uninjured leaves

100.9 - 0.94x
FIGURE 6. Effect of *T. urticae* feeding injury on peppermint leaf weight (EXP. I). The Y-axis is the ratio of leaf weights of injured to uninjured leaves, expressed as a percent \(((\text{injured/uninjured})\times100)\). The X-axis is the mite-days index — mite density \(\times\) duration of feeding (days). Unlabelled points represent the means of 4 replicates. Labelled points represent the following numbers of replicates: a=47, b=3, c=12, d=8. \(100.6 - 0.14X\), \(r^2 = 0.54\), \(P < .001\), \(n=173\). The mean leaf weight of uninjured leaves = 0.0854 grams (SD = 0.023).
LEAF FRESH WEIGHT
EXP. 1

Mite-Days

Figure 6
FIGURE 7. Effect of *T. urticae* feeding injury on peppermint leaf area (EXP. II). The Y-axis is the ratio of injured to uninjured leaves leaf area, expressed as a percent \(((\text{injured/\text{uninjured}})\times100)\). Each point represents the mean of 4 replicates. \(100.9 - 0.36X\), \(r^2 = 0.06\), \(P=.01\), \(n=112\). The mean area of uninjured leaves = 15.05 cm\(^2\) (SD = 3.79).
Figure 7

LEAF AREA

Leaf Area (% of uninjured leaves)

- 105
- 100
- 95
- 90

Mite-Days·cm⁻²

- 0
- 5
- 10
- 15
- 20

uninjured leaves

100.9 - 0.36x

LEAF AREA
shows that leaf area decreased by ca. 0.36% with each increment of injury. While the coefficient of determination is low in this case ($r^2 = 0.06$), a statistically significant relationship is nonetheless suggested (slope of regression line $\neq 0; P = .01$).

The data of Figures 5 and 7 have been combined as specific weight (grams per unit area) in Figure 8. Specific leaf weight remained relatively unchanged until high injury levels were reached.
FIGURE B. Effect of *T. urticae* feeding injury on specific weight (grams/cm²) of peppermint leaves (EXP. II). The Y-axis is the ratio of specific leaf weights of injured to uninjured leaves ((injured/uninjured)×100). Each point represents the mean of 4 replicates. 96.37 + 1.43X - 0.191X² + 0.004X³; r² = 0.35, n=112. The mean specific weight of uninjured leaves = 0.012 grams/cm² (SD = 0.0011).
Figure 8

SPECIFIC WEIGHT

Specific Weight (gm·cm⁻²)
(% of uninjured leaves)

Mite-Days·cm⁻²

uninjured leaves
DISCUSSION

According to equation 1, the flux of water vapor per unit leaf area is proportional to the difference in water vapor concentration between mesophyll cell wall pores and the bulk, turbulent air surrounding the leaf, and inversely proportional to total resistance to diffusion of the air boundary layer and leaf. Stomatal movements exercise control over only one part of the diffusion pathway and therefore their effects will vary with the magnitude of the boundary layer and cuticular resistances relative to that of stomatal resistance. For example, with the stomates fully open the leaf behaves nearly as a free water surface with respect to water vapor flux per unit area, and decreasing cuticular or boundary layer resistance would have little effect on transpiration. On the other hand, when stomatal resistance is high because of stomatal closure, a small decrease in cuticular resistance can mimic open stomates and prevent reduction in transpiration to the degree normally associated with stomatal closure.

The increase in leaf conductance (decreased leaf resistance) that was observed at night in mite-injured peppermint leaves (Fig. 3), probably resulted from a decrease in cuticular resistance due to feeding damage to
the leaf epidermis and cuticle. Duniway and Durbin (1971a, 1971b) observed the same phenomenon in snapbean leaves infested with rust fungus. Rusted leaves transpired significantly more intensely at night than did healthy leaves and Duniway and Durbin attributed the difference to a lower cuticular resistance of infected leaves. Tanigoshi and Davis (1978) observed that spider mite-injured apple leaves exhibited disrupted epidermal cells, and Sances et al. (1979a) found groups of epidermal cells to be misshapen and collapsed in mite-injured strawberry leaves. The possibility remains, however, that spider mite feeding injury might inhibit stomatal closure at night and thereby maintain rates of transpiration that approach daytime levels.

The observation that transpiration (leaf conductance) was lower during the day in injured leaves (Fig. 2) argues against the latter interpretation. If stomatal closure were inhibited by feeding injury to the extent necessary to bring about the observed nighttime transpiration rates (Fig. 3), one would expect that stomatal response during the day would likewise be inhibited and that the reduction in transpiration that was found (Fig. 2), would not have been observed.

The decrease in leaf conductance (increased leaf resistance), measured during the day in injured leaves (Fig.
2), was probably the result of partial stomatal closure in response to water stress. Sances et al. (op. cit.) reported that 61% of stomata were closed in spider mite-injured strawberry leaves. It has been well documented that developing water stress can cause stomatal closure, and for some plants this response is highly sensitive to small water deficits (Hsiao 1974). De Santo (1973) found that peppermint actively controlled water loss under conditions of high evaporative demand, presumably by stomatal closure.

Water loss through damaged epidermal cells and cuticle during the previous night may account for the observed lowering of leaf osmotic potential the following day (Fig. 4). Plants generally take advantage of favorable nighttime conditions (low evaporative demand and high leaf resistance) to replenish leaf water lost during the day. Root resistance to liquid water transport places an upper limit on water uptake capacity (Nobel 1974), and may, in conjunction with continued nighttime transpiration, prevent injured leaves from fully regaining water lost during the previous day.

Barrs and Klepper (1968) demonstrated that cyclic fluctuations in stomatal aperture, transpiration rate, and leaf water potential in plants kept under constant environmental conditions could be accounted for on the basis of diurnally varying root resistance to liquid water.
transport. Root resistance was minimal at midday and gradually increased to a maximum near midnight (Barrs and Klepper op. cit.). These findings suggest that high nighttime resistance to liquid water flow across roots may indirectly contribute to development of water stress during the day in mite-injured leaves. Roots have been identified as the site of major resistance to water flow in the soil-plant-atmosphere system (Barrs and Klepper op. cit., Blizzard and Boyer 1980). In fact, the resistance to flow offered by the plant was found to be always higher than soil resistance over the entire range of soil water availability normally encountered (Blizzard and Boyer op. cit.). The species specific root to shoot ratio may then have important consequences relating to a plant's ability to withstand spider mite feeding damage by influencing water uptake capacity at night. Plants with higher root:shoot ratios were less influenced by high root resistance than were plants with lower ratios, with regard to the induction of spontaneous, cyclic rhythms (Barrs and Klepper op. cit.).

In other studies (unpublished data; see Chapter V), spider mite-injured peppermint leaves were found to have significantly higher concentrations of soluble carbohydrates than uninjured leaves. The magnitude of the difference was correlated with degree of injury. An osmotic adjustment mechanism, responding to leaf water deficits and utilizing
soluble carbohydrates to maintain leaf turgor, may be responsible for lowered leaf osmotic potentials. Osmotic adjustment, defined as net solute accumulation (Turner et al. 1978), has been observed in a number of plant species in response to water deficits (Hsiao 1973, Begg and Turner 1976, Jones and Turner 1978, Turner et al. op. cit.).

_Tetranychus urticae_ feeding injury significantly affected leaf fresh weight in the present studies (Figs. 5 and 6). Leaves used in EXP. I had not yet fully expanded, and were considerably smaller than leaves used in EXP. II (0.085 versus 0.185 grams mean fresh weight, respectively). However, both experiments yielded similar results suggesting that weight loss was probably not the result of growth inhibition. A more likely explanation is that the observed weight loss was directly related to leaf water deficits.

Reductions in leaf area associated with spider mite injury have been reported for almond leaves (Summers and Stocking 1972). Results of the present studies suggest that once peppermint leaves are fully expanded, leaf area is relatively insensitive to mite injury (Fig. 7). Leaves not fully expanded, however, appeared to be much more sensitive to injury (EXP. I.). Although Summers and Stocking (op. cit.) reported 30% reduction in leaf area of heavily damaged leaves, the degree of injury and the duration of injury were much higher in their studies. Specific leaf weight did not
change significantly until high injury levels were reached (Fig. 8). The injury level at which specific leaf weight began to drop sharply (ca. 10 mite-days/cm²) may mark the critical injury threshold for peppermint. At this point, reductions in leaf area could no longer compensate for weight loss (water loss) and marks the beginning of a phase of rapid water loss, eventually resulting in leaf death.

Many of the effects of spider mite feeding injury on peppermint leaf water relations, particularly water deficits, can probably be accounted for on the basis of mechanical damage to epidermal cells and cuticle. Increased water loss through these damaged protective layers, especially at night, causes leaf water deficits the following day, reduced daytime gas exchange and hence reduced photosynthesis (see Chapter 4), and may, if young leaves are attacked, reduce the leaf area available for photosynthesis.
Effects of *Tetranychus urticae* Koch Feeding Injury on Physiological Processes in *Mentha piperita* L.

CHAPTER III

Sequential Extraction and Analysis of Peppermint Tissue
New methods for sequential extraction of peppermint (Mentha piperita L.) leaf, stem, and rhizome tissues are presented. These methods have proven to be both effective and reliable in investigations of effects of spider mite feeding injury on host plant physiological processes. The methods enable sequential extraction of terpenes of peppermint oil, soluble carbohydrates, leaf starch, and leaf photosynthetic pigments, from very small amounts of fresh tissue (50-500 mg). A new method of in situ leaf starch hydrolysis also is presented.
INTRODUCTION

Published methods of plant tissue analysis, and methods for analysis of plant secretory products, have usually dealt with one or a few chemically related compounds (cf. Dekker and Richards 1971, Farley and Howland 1980, Gorin and Heidema 1980, Tella and Ojehomon 1980, Lane and Schuster 1981). It is often necessary in physiological investigations, however, to analyze for several groups of chemically distinct compounds from the same tissue sample. These samples may be quite small--a gram or less, fresh weight. In such cases, reliable sequential extraction schemes are needed. Recent advances in analytical instrumentation (e.g. GC, HPLC, GC-MS) have simplified quantitative microanalysis of plant compounds, but sample preparation and fractionation continue to present problems.

The need for specific tissue extraction and analysis methods was recognized early in investigations of biotic factors affecting terpene biosynthesis in peppermint (Mentha piperita L.). Methods were required that would allow for extraction and quantification of 1) terpene components of peppermint oil, 2) soluble carbohydrates (mono-, di-, and
oligosaccharides), 3) leaf starch, and 4) photosynthetic pigments -- from single, 50-150 mg fresh weight samples of leaf, stem, or rhizome tissue. An additional requirement was that each step in the extraction and fractionation process be as gentle as possible in order to minimize effects of one step on subsequent steps in the procedure.

This paper presents new methods for sequential extraction of peppermint tissues. Included also is a description of the analytical methods used throughout studies designed to investigate the effects of spider mite feeding injury on host plant physiological processes.
METHODS AND MATERIALS

PLANT MATERIAL. Peppermint plants (var. Black Mitcham, Willamette Valley clone) were grown from field-collected rhizomes in beds of horticultural grade vermiculite. Beds were cut back at approximately three week intervals to maintain vigorous vegetative growth (Crane and Steward 1962). Plants were periodically watered with a modified Hoagland-Arnon nutrient solution (Hoagland and Arnon 1938, as modified by Crane and Steward, op. cit.). Ambient conditions in the growth chamber were maintained at 16 hr light; 23-25°C day/20-22°C night temperature regime; 40-60% relative humidity.

GENERAL EXTRACTION SEQUENCE. The essential features of the extraction sequence are shown in Figure 9. Various fractions were collected at points in the sequence and these were stored as necessary.

Fresh plant material was first weighed on an analytical balance (Mettler H10). Leaves were then submerged in 4 or 5 ml of cold (0°C) 30% aqueous ethanol (30% EtOH) in 12 ml conical, thick-walled centrifuge tubes. Stem and rhizome tissue was first ground in liquid nitrogen, and the resulting powder transferred to centrifuge tubes. Four or 5
**TISSUE SAMPLE-30% AQUEOUS ETHANOL EXTRACT**

4 or 5 ml 30% EtOH / 50-500 mg plant material (fresh weight), extract 30 min at 00 °C, centrifuge 1200 × g for 10 min (if necessary), reextract with 4 or 5 ml 30% EtOH for 20 min at 00 °C, centrifuge as before and combine extracts in ice bath.

### All Tissue
- **30% Aqueous EtOH Extract (0-10 ml)**
  - add ca. 1 ml pentane, mix by inversion (10 inversion cycles), let stand 10-15 min in ice bath, remove pentane, repeat with an additional 1 ml pentane, combine pentane fractions.

### Leaf Tissue Only
- **Insoluble Tissue Residue**
  - add 16 ml acetone, let stand 2-4 hr in darkened cabinet at room temp. with occasional vortex mixing, dilute to 80% acetone with distilled water, dilute 1 ml aliquots with 4 ml 80% acetone, determine absorbance at 652 nm.

### Insoluble Tissue (Pigment free)
- add 9 ml 0.01 M acetate buffer, pH 4.5, cap and place in boiling water bath for 1 hr, cool, add 1 ml amylolglucosidase reagent*, incubate 20-24 hr at 50°C.
  - glucose derived from starch

### Neutral Fraction
- add distilled water to constant volume, pass extract through resin column - top layer: 5 ml PVPP; bottom layers 15 ml of a 1:1 (w/w) mixture of IR-45 (formate) and XAD-4, elute columns with distilled water.

### Pentane Fraction
- evaporate pentane down to constant volume under dry nitrogen stream
  - volatile oil components (terpenes)

### Insoluble Tissue Residue
- add 16 ml acetone, let stand 2-4 hr in darkened cabinet at room temp. with occasional vortex mixing, dilute to 80% acetone with distilled water, dilute 1 ml aliquots with 4 ml 80% acetone, determine absorbance at 652 nm.

### Insoluble Tissue (Pigment free)
- add 9 ml 0.01 M acetate buffer, pH 4.5, cap and place in boiling water bath for 1 hr, cool, add 1 ml amylolglucosidase reagent*, incubate 20-24 hr at 50°C.
- glucose derived from starch

### Amyloglucosidase reagent
- 24 units of amylolglucosidase (Sigma Chemical Co.) / 1 ml 0.01 M acetate buffer, pH 4.5.

---

* Samples may be stored frozen at these points.

** Samples may be stored cold (-20 to 70°C) if steps are taken to prevent loss of pentane from sample tubes.

** Amyloglucosidase reagent - 24 units of amylolglucosidase (Sigma Chemical Co.) / 1 ml 0.01 M acetate buffer, pH 4.5.

---

Figure 9
ml of cold 30% EtOH was then added to each tube containing ground tissue and the contents mixed with a glass stirring rod.

Extraction was carried out at 80°C for 30 min in a water bath. Centrifuge tubes were stoppered with neoprene stoppers wrapped with aluminium foil. Aluminium foil wraps were necessary to prevent adsorption of terpenes onto the neoprene stoppers. This precaution was particularly important in preventing loss of the lower boiling point terpenes (unpublished data). Following the first extraction, leaves were removed from their tubes and carefully transferred to a second set of tubes again containing cold 30% EtOH. In the case of ground tissue, tubes were centrifuged (ca. 1200 x g, 10 min.), the supernate was removed, and an equal volume cold 30% EtOH was added. A second extraction was carried out at 80°C for 20 min. Following the second extraction and centrifugation (when necessary), the first and second extracts were combined. The residue tissue was then stored frozen in distilled water for later analysis.

One ml of reagent grade pentane (Baker Resi-Analyzed) was added to the combined 30% EtOH extracts. Extract and pentane were mixed by careful inversion of the tubes (ca. 10 inversion cycles), then returned to the ice bath. After the phases had completely separated (ca. 15 min.), the pentane
fraction containing the terpenes was removed with a Pasteur pipette. The terpene fractionation was then repeated with an additional 1 ml of pentane as before. If not immediately analyzed, pentane fractions were stored at -70°C in tightly sealed glass tubes. The remaining terpene-free 30% EtOH extracts were quantitatively transferred to 50 ml polypropylene centrifuge tubes and stored frozen (-20°C).

COLUMN CLEAN-UP. Plant extracts generally require treatment to remove substances that interfere with analytical methods (Loomis and Battaile 1966, Andersen and Sowers 1968, Anderson 1968, Lam and Shaw 1970, Loomis 1974, Loomis et al. 1979). These compounds commonly are phenolics, quinones, and organic acids. The 30% EtOH fractions were passed through mixed-bed resin columns (30 ml polypropylene syringe barrels) in order to remove these potentially interfering compounds. Each column contained 10 ml of a 1:1 mixture of IR-45 (Rohm & Hass), a weakly basic anion exchange resin, and XAD-4 (Rohm & Hass), a non-ionic polymeric adsorbent. Five ml of PVPP (polyvinylpolypyrrolidone) was layered on this resin bed. The anion exchange resin was conditioned just prior to use by batch and column treatment with 1N formic acid and 2M sodium formate. All resins were thoroughly washed upon receipt from the manufacturers. The washing procedure included suspending the resins in
distilled water, methanol, 3N HCL, and again in distilled water until free of chloride ion. The neutral fraction (Fig. 9) was eluted from the columns with distilled water.

PIGMENT AND STARCH EXTRACTION. The distilled water in which tissue residues had been stored was drawn off by aspiration. Sixteen ml of acetone was added to samples of leaf tissue. Extraction of leaf pigments was carried out in a darkened cabinet for 2-4 hours at room temperature. Water was then added to the extracts to a final concentration of 80% acetone. A 1 ml aliquot of this extract was then diluted with 4 ml of 80% acetone, and the absorbance determined at 652 nm (Arnon 1949, Bruinsma 1963).

Leaf starch was determined on the remaining tissue residue. Starch was analyzed as glucose equivalents after in situ enzymatic hydrolysis with amyloglucosidase (Sigma Chemical Co.). Samples were first heated (100°C) in 0.01 M acetate buffer, pH 4.5, for 60 min in tightly stoppered culture tubes. After cooling the tubes to room temperature, 1 ml of amyloglucosidase reagent (ca. 24 units, Fig. 9) was added to each tube and the mixture incubated at 45°C for 20-24 hr. The resulting glucose was determined on 0.5-1 ml aliquots of the hydrolysate by the glucose oxidase-peroxidase-o-dianisidine (GOX) method (Teller 1956, MacRae 1971, Haissig and Dickson 1979).
ANALYTICAL METHODS. The terpenes of the pentane fraction were analyzed by gas chromatography as described in the caption for Figure 10.

Soluble carbohydrates of the neutral fraction (Fig. 9) were analyzed as reducing sugar equivalents after acid hydrolysis. An aliquot of each neutral fraction was acidified with 5 N sulfuric acid to a final acid concentration of 1 N. Acidified samples were then heated at 100°C for 60 min, cooled, and neutralized with 5 N sodium hydroxide. This treatment is sufficient to hydrolyze di- and oligosaccharides completely to their component reducing monosaccharides without danger of further degradation (McCready 1970). The resulting reducing sugar equivalents were quantified by the Somogyi-Nelson reducing sugar method (Somogyi 1938, Nelson 1944, Somogyi 1945). This method is sensitive to the presence of non-carbohydrate reducing substances (e.g. ascorbic acid, phenolic acids); however, after column treatment of the extracts as described above, this interference was reduced to undetectable levels.

Thin-layer chromatography (TLC) was used for qualitative determinations of the 30% EtOH soluble carbohydrates of peppermint leaf, stem, and rhizome tissues. Ascending development on cellulose plates (Kodak Chromagram sheets) with n-butanol:ethyl acetate:acetic acid:water
(4:3:2.5:4 v/v) as the solvent system was used.
Carbohydrates were visualized with aniline-diphenylamine spray reagent (Sigma Chemical Co.), used according to the manufacturer's recommendations.
Figure 10.- GC CHROMATOGRAM OF PENTANE FRACTION. Peppermint leaf sample (0.097 g fresh weight). Sample volume = 0.2 ml; 0.05 ml injected with a 10:1 split ratio. Hewlett Packard 5710 A gas chromatograph equipped with flame ionization detector. Column: 0.2 mm x 50 m flexible silica W.C.O.T. capillary, Carbowax 20M coated. Carrier gas flow through the column 1 cc/min helium, with make-up to the detector at 30 cc/min, hydrogen at 30 cc/min, air at 220 cc/min. Peak area integration done on an HP 3380 A reporting integrator using internal standard method of calibration. Temperature programming: 16 min isothermal at 70°C, 2°C/min to 120°C and held for 4 min. Injection port temp. 250°C, detector temp. 300°C. The amount of n-tridecane internal standard in this run was 40 ng. Labelled peaks account for 95% of the extracted oil.
Figure 10
RESULTS AND DISCUSSION

Thirty percent aqueous ethanol proved to be a superior extracting solvent for peppermint tissues. Soluble carbohydrates and terpenes of bean leaf and stem tissue, and carbohydrates of fresh and dried root crown tissue from tansy ragwort (Senecio jacobaea), also have been successfully analyzed using this extraction method (unpublished data). Thirty percent ethanol was chosen as the extracting medium because: 1) even slightly higher concentrations of alcohol solubilize and extract plant pigments which then must be eliminated from the extract in subsequent steps; 2) lower alcohol concentrations at elevated extraction temperature, solubilize and extract amylase, resulting in an underestimation of starch (Table 1); 3) extraction in 30% EtOH facilitates extraction of essential oils (terpenes) because many lower molecular weight terpenes are miscible in alcohol, but insoluble in water; and 4) terpenes are easily recovered from the 30% EtOH by pentane fractionation, resulting in very clean pentane fractions that require no further treatment prior to
Table 1.- SOLUBILITY OF STARCH IN AQUEOUS ETHANOL. Non-soluble potato starch was heated in aqueous EtOH, 45 min, 80°C. The solutions were then cooled and centrifuged, and the supernate was analyzed for starch by iodine precipitation followed by hydrolysis with amyloglucosidase. Glucose measured by the glucose oxidase method (n=3).

<table>
<thead>
<tr>
<th>% Ethanol (v/v)</th>
<th>% Starch Solubility (as recovered glucose, $\bar{X} \pm SD$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.0 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>30</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>
gas chromatographic (GC) analysis. Recoveries of exogenous oil from 30% EtOH by pentane fractionation were found to be 93.1% (n=3). Recoveries of α- and β-pinene, limonene, β-caryophellene, and germacrene D were less (40–65%); however, these terpenes constitute less than 10% of the terpenes of peppermint oil.

Samples eluted from the resin columns were slightly acidic (pH ca. 3.5). Eluted samples were clear, and remained clear even after prolonged storage, indicating that the browning reactions characteristic of plant phenolic compounds were absent. No interference of analytical procedures for carbohydrates has thus far been detected from samples prepared in this way. Samples eluted from the resin columns have a final alcohol concentration of ca. 8%. Recoveries of standard glucose and sucrose solutions from the columns exceeded 95% for both sugars.

Figure 11 shows the time-course of leaf starch hydrolysis by amyloglucosidase. Hydrolysis was essentially complete after 20 hr incubation. The slight decline in recovered starch after 48 hr of hydrolysis (Fig. 11) was probably the result of microbial contamination. Completeness of starch hydrolysis and recovery of starch-derived glucose were investigated. Fifteen peppermint leaf samples were selected at approximately midway through a light period, processed as outlined in Fig.
FIGURE 11.—TIME COURSE OF IN SITU LEAF STARCH HYDROLYSIS.
Rate of hydrolysis of leaf starch by amylglucosidase. Each point is the mean of 3 leaf samples. Vertical lines represent 1 standard deviation. Samples were treated as described in Fig. 9. Glucose determined by glucose oxidase method.
IN SITU LEAF STARCH HYDROLYSIS

Figure 11

mg Starch gm Fresh Weight$^{-1}$ vs. HOURS
<table>
<thead>
<tr>
<th>TISSUE TYPE</th>
<th>LEAF</th>
<th>STEM</th>
<th>RHIZOME</th>
<th>$R_{\text{fructose}}^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRUCTOSE</td>
<td>FRUCTOSE</td>
<td>FRUCTOSE</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>SUCROSE **</td>
<td>SUCROSE</td>
<td>SUCROSE</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>RAFFINOSE</td>
<td>RAFFINOSE **</td>
<td>RAFFINOSE</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>STACHYOSE</td>
<td>STACHYOSE **</td>
<td>STACHYOSE</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>VERBASCOSO $^2$</td>
<td>VERBASCOSO **</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R VI **</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R VII **</td>
<td>0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R VIII **</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R IX</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R X</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 12.- SOLUBLE CARBOHYDRATES OF PEPPERMINT BY TISSUE TYPE.

$^1$Relative mobilities on cellulose (Kodak Chromagram) thin-layer plates compared to mobility of fructose. Solvent: n-butanol:ethyl acetate:acetic acid:water (4:3:2.5:4).

$^2$Verbascose identified by relative mobility only.

** Indicates predominant carbohydrates of each tissue type.

Compounds R VI - R X are unknown.
9 for leaf starch. Average starch content was found to be 7.75 mg per gram fresh weight. Following the normal starch analysis procedures, the 15 samples were randomly grouped into 5 samples of 3 leaves each. The 3 leaves in each group were carefully removed from the original tubes, combined, and ground in liquid nitrogen to a fine powder. These ground samples were then reprocessed in the normal manner (Fig. 9), except the pentane fractionation step was omitted. Ethanol extracts of the ground tissue contained less than 1% (0.6%, n=5) of the starch-derived glucose of the original samples, indicating that an equilibrium had been established in the original hydrolysis between glucose in the tissue and in the surrounding solution. No additional glucose was detected following amyloglucosidase hydrolysis of the combined and ground tissue samples.

Figure 12 shows the distribution of 30% EtOH soluble carbohydrate of peppermint leaf, stem, and rhizome tissues. Experiments are currently underway to isolate and identify the five unknown oligosaccharides (R VI - R X, Fig. 12) of rhizome tissue by gel filtration chromatography. Preliminary evidence suggests that fructose is an important constituent of these polymers.

Peppermint oil extraction has generally been done in one of two ways—steam distillation of whole, air-dried plants, or direct extraction of fresh, ground tissue with
organic solvents. Commercial processors use steam distillation because large amounts of plant material can be processed quickly and cheaply. Steam distillation has also been used in the laboratory, but it is impractical to attempt to scale down this method for single leaf samples. Direct extraction of fresh, ground tissue with organic solvents (usually hexane) has been used successfully on small samples (Burbott and Loomis 1967, 1969). The one important disadvantage of this method is that the ground tissue residue remaining after hexane extraction, is not easily subjected to additional analytical procedures (e.g. soluble carbohydrate and starch analysis). In addition, the direct extraction method is relatively time-consuming compared to the present method.

Preliminary experiments were conducted to compare the direct extraction method (Burbott and Loomis 1967, 1969) and the 30% EtOH extraction method presented here (Fig. 9), with regard to recovery of terpenes from single peppermint leaves. Results suggested that these methods are comparable in efficiency of extraction of peppermint oil terpenes. Some loss of lower boiling point monoterpene hydrocarbons was experienced with the 30% EtOH method, but these are only very minor constituents of the oil. Sodium sulfate used in the direct extraction method was shown to adsorb ca. 15% of some terpenes (unpublished data). In summary, while
recovery of peppermint oil terpenes from fresh tissue samples was found to be entirely adequate using either method of extraction, the constraints of studies in which additional analyses are required on the extracted tissue make the 30% EtOH extraction method a more viable alternative.
Effects of Tetranychus urticae Koch Feeding Injury on Physiological Processes in Mentha piperita L.

CHAPTER IV

Effects on Photosynthesis and Leaf Chlorophyll Content
ABSTRACT

Studies were conducted to examine the effects of feeding injury by *Tetranychus urticae* Koch (Acari:Tetranychidae) on photosynthesis in peppermint, *Mentha piperita* L. The most detrimental effects were loss of leaf chlorophyll through disruption of mesophyll cells, and reduction in carbon dioxide influx resulting from lowered leaf conductance. The rate of photosynthesis was correlated with leaf conductance, and the relationship was unaffected by mite injury. Chlorophyll loss is discussed in terms of its possible use as a index of cell disruption.
INTRODUCTION

Spider mites (Acari:Tetranychidae) are extremely destructive plant pests in both annual and perennial agricultural systems (Briggs and Avery 1968, Huffaker et al. 1969, Golik 1975, Jeppson et al. 1975). *Tetranychus urticae* Koch, the twospotted spider mite, is one of the most widely distributed and destructive species in agricultural situations. Mites of the *T. urticae* species complex have been recorded from more than 150 host plants of economic value, including most of the important agricultural crops and ornamental plants (Jeppson et al. op. cit.).

Spider mites feed primarily on leaf surfaces, often occurring in higher numbers on the undersides than on the tops of leaves. Injury results from the mite probing through the leaf surface with long (100-150μm), narrow cheliceral stylets, and removing the contents of mesophyll cells (Jeppson et al. 1975). Probing causes collapse of epidermal cells and disruption of overlying cuticle (Tanigoshi and Davis 1978, Sances et al. 1979a). Penetrated mesophyll cells are generally devoid of cytoplasm, but cell walls are not removed in the feeding process (Summers and Stocking 1972, Tanigoshi and Davis op. cit.). In addition
to mechanical injury, leaves may be subjected to the action of toxic or growth-regulating substances, injected along with saliva, that may contribute to the general response of plants exposed to spider mite feeding injury (Avery and Lacey 1968, Stormes 1972).

Much of the early research concerned with effects of spider mite feeding injury on host plant physiology dealt with effects on photosynthesis and transpiration (Boulanger 1958, Wedding et al. 1958, Atanasov 1971, Kolodziej et al. 1974, Golik 1975, Hall and Ferree 1975). The most important conclusions drawn from these studies were that, when compared to healthy leaves, 1) severely injured leaves exhibited lower rates of photosynthesis and transpiration; 2) low to moderate injury sometimes resulted in increased transpiration; 3) injured leaves were often smaller or otherwise deformed; and 4) the chlorophyll content was usually found to be less in injured leaves. More recent studies, designed to investigate the underlying mechanisms of spider mite injury, have suggested that effects on leaf gas exchange and cellular ultrastructure may be the most important detrimental effects of spider mite feeding injury (Summers and Stocking 1972, Tanigoshi and Davis 1978, Sances et al. 1979a, 1979b). Tanigoshi and Davis (op. cit.) demonstrated fine structural alterations in chloroplasts of cells adjacent to, but not directly involved with, feeding
sites of Tetranychus mcdanieli McGregor on apple. Sances et al. (1979b) found that T. urticae disrupted the functional integrity of epidermal and stomatal guard cells and that this resulted in changes in transpiration and photosynthesis of affected leaves.

The study reported here examined the effects of T. urticae feeding injury on photosynthesis in peppermint, Mentha piperita L. Assimilation of carbon-14 was used to monitor relative rates of photosynthesis in variously injured, and uninjured, leaves. Chlorophyll determinations were made to assess the magnitude of cellular disruption at each injury level. Effects on gas exchange (Chapter II) are discussed in terms of the photosynthetic processes in injured leaves.
METHODS AND MATERIALS

Two separate series of experiments were conducted, designated EXP. I and EXP. II. The series were similar in design, with minor differences as noted in Chapter II. EXP. I was primarily intended to provide preliminary information on which later experiments could be based. The methods and materials used in the culturing of experimental plants and in the maintenance and culturing of the Tetranychus urticae colony are presented elsewhere (Chapter II).

EXPERIMENTAL DESIGN. Details of the experimental design of EXP. I and EXP. II are presented in Chapter II, along with background information regarding the use of the mite-days/cm² injury index. Therefore, only a brief synopsis will be presented here.

One leaf of a pair of peppermint leaves on each plant was infested with virgin female T. urticae at rates of 2, 5, 10, or 20 mites per leaf. The uninfested leaf of the pair served as an intraplant control. Determinations of photosynthesis and leaf chlorophyll, as well as other parameters associated with studies reported on in Chapters
FIGURE 13. Method of calculating the mite-days/cm$^2$ injury index used in these studies. "COUNT" refers to the number of living adult female *T. urticae* at the beginning (initial infestation level) of the experiment (N=0) and at each sampling day (N).
Mite-Days \( \div \text{cm}^2 \) =

\[
\left( \frac{\text{COUNT}_0 + \text{COUNT}_N}{2} \right) \cdot N
\]

\[ \text{AREA OF UNINFESTED LEAF (cm}^2) \]

where: 0 & N = DAYS AFTER INFESTATION

Figure 13
II and V, were made on paired leaves sampled at 0, 2, 4, 6, 8, 10, 12, 14, and 16 days (EXP. I), or 3, 6, 9, 11, 13, 15, and 17 days (EXP. II) after infestation. Each day and mite density combination was replicated 4 times for totals of 360 samples in EXP. I and 224 samples in EXP. II.

The Mite-Days/cm²injury index was calculated as shown in Figure 13. All measured parameters in EXP. II were scaled against this index in order to account for variation within treatments in leaf size and mite populations.

LEAF CONDUCTANCE. Leaf conductance measurements were made using the LiCor LI-65 Autoporometer (LiCor Inc., Lincoln, Nebraska) as described in Chapter II. Leaf conductance was calculated as the reciprocal of total leaf diffusive resistance (Chapter II). Leaf conductance measurements were made just prior to photosynthesis measurements.

14-CO₂ PHOTOSYNTHESIS. Photosynthesis in injured and uninjured peppermint leaves was measured by 14-carbon dioxide (14-CO₂) assimilation. This method is based on applying short pulses of air containing 14-CO₂ over a small area of leaf enclosed within a chamber. The rate of photosynthesis is assumed to be proportional to the amount of labelled carbon assimilated per unit time. The method has been widely used under both laboratory and field

The apparatus for labelling leaves consisted of 3 components: 1) a "field" compressed air tank containing labelled CO and air; 2) a system for introducing 14-CO₂ into the field tank and filling the tank with air at high pressure; 3) an assimilation chamber and air delivery system for the actual labelling process. Each component will be described below.

The field tank was made from a 500 cm³ stainless steel high pressure cylinder (Whitey Co., Cleveland, OH). The system for filling it with labelled air is shown schematically in Figure 14. The entire system was first sealed and then evacuated by means of a vacuum pump. 14-CO₂ was generated by introducing 1N acetic acid into the reaction chamber through the serum stopper-sealed port. The reaction chamber contained a measured amount of 14-C labelled (7.5 mCi/mmol) sodium bicarbonate (New England Nuclear). The entire system was pressurized by opening the valve from the high pressure air tank. Pressurization was done slowly, in small increments, to avoid adiabatic heating of the field tank. The field tank was pressurized to 800-1500 psi, depending on the number of samples anticipated.

The assimilation chamber was similar in design to the
FIGURE 14. System for filling field tank (1) with labelled air. Field tank is connected to the reaction chamber (2), vacuum pump (3), and high pressure air tank (4) through 3/8" heavy-walled aluminium tubing (c). A brass check valve (a) controls air flow from the field tank. Valves (b) are situated between the reaction chamber, vacuum pump, and high pressure tank for isolating the system. The reaction chamber top is sealed to the body by bolts and an "O" ring (d). Labelled sodium bicarbonate is placed in the reaction chamber in a small beaker (f). Acid is added through a serum stopper-sealed port (e).
one described by Naylor and Teare (1975), except that the solenoid that controlled air flow through their chamber was replaced by a hand-operated toggle valve. Air flow through the chamber was monitored by means of a flow meter (Union Carbide Corp.) situated between the field tank and assimilation chamber.

Leaf disc sample preparation for liquid scintillation counting followed the procedures of Mahin and Lofberg (1966). An initial attempt at wet tissue digestion with strong base (TS-1 tissue solubilizer, RPI Corp.) was unsuccessful and resulted in samples that were so highly color quenched that they could not be counted. Because of this technical difficulty, no photosynthesis data are available from EXP. I. Quench correction for liquid scintillation counting was by the channels ratio method, according to the manufacturer's recommendations (ISOCAP 300 Liquid Scintillation Counter, Nuclear Chicago). Typical counting efficiency for the sample preparation method of Mahin and Lofberg (op. cit.) was ca. 60%. 14-C Toluene (4.1 x 10^10 dpm/ml, New England Nuclear) was used as standard for calibration and quench correction purposes.

Chlorophyll was extracted and analyzed according to the methods of Arnon (1949) and Bruinsma (1963), and as described in Chapter III.
RESULTS AND DISCUSSION

The results of this study do not differ significantly from the results of similar studies conducted by other workers on other spider mite/host plant systems with regard to the response of photosynthesis to mite injury (Wedding et al. 1958, Atanasov 1971, Hall and Ferree 1975, Sances et al. 1979b). The rate of photosynthesis was lower in mite-injured leaves (Figure 15), and the magnitude of the difference between injured and uninjured leaves was correlated with injury when estimated by the mite-days/cm² index (Fig. 13).

Figures 16 and 17 show the loss of leaf chlorophyll with increasing spider mite injury. The data from EXP. I are shown in Fig. 16. Chlorophyll loss was estimated to be ca. 0.14% per mite-day increment of injury (Fig.16; \( r^2 = 0.42 \)). The rate of loss was a linear function of injury suggesting that feeding rate was essentially constant throughout the experimental period (see below). At the highest injury level (320 mite-days), injured leaves had experienced ca. 40% loss in chlorophyll (Fig. 16). Figure 17 shows the data from EXP. II. In this case, injury was
FIGURE 15. Effect of T. urticae feeding injury on 14-CO$_2$ assimilation in peppermint. The Y-axis is the ratio of the photosynthetic rates of injured versus uninjured leaves, measured as disintegrations per minute (DPM) of each sample leaf disc ((injured/uninjured)x100). The X-axis is the mite-days/cm$^2$ index, calculated as shown in Fig. 13. Each point represents the mean of 4 replicates. n=81. log Y = 1.998 - 0.0137 X. r$^2$ = 0.33.
Figure 15

$^{14}\text{CO}_2$ Assimilation

DPM (% of uninjured leaves)

Mite-Days cm$^{-2}$
FIGURE 16. Effect of *T. urticae* feeding injury on chlorophyll content of leaves (EXP. I). The Y-axis is the ratio of injured to uninjured leaves \(((\text{injured/uninjured}) \times 100)\). The X-axis is the mite-days index, calculated as mite density \(\times\) duration of feeding (days). Letters refer to the number of replicates represented by each point: a=32, b=3, c=11, d=12, e=8, unlettered points = 4. \(n=156, 96.2 - 0.136 X; r^2 = 0.42; p<.001\).
Figure 16

CHLOROPHYLL a+b

EXP. I

uninjured leaves

Mite-Days

Chlorophyll a+b (gm-leaf⁻¹)
(% of uninjured leaves)
FIGURE 17. Effect of T. urticae feeding injury on chlorophyll content of leaves (EXP. II). The Y-axis is the ratio of injured to uninjured leaves \(((\text{injured/uninjured}) \times 100)\). The X-axis is the mite-days/cm² index, calculated as shown in Fig. 13. Each point represents the mean of 4 replicates. \(n=112\), \(97.55 - 2.38 X + 0.063 X^2\); \(r^2 = 0.46\).
Figure 17

CHLOROPHYLL a + b

Chlorophyll a+b (% of uninjured leaves) vs. Mite-Days cm⁻²

uninjured leaves
estimated by the mite-days/cm² index.

If the assumption is made that loss of extractable chlorophyll in mite-injured leaves is primarily the result of cell disruption and removal of protoplasts, and not caused by enhanced metabolic degradation or decreased synthesis, then chlorophyll loss may be used as a measure of the number of cells destroyed at each injury level. Water stress has been shown to substantially reduce chlorophyll accumulation in etiolated leaves (Virgin 1965); however, chlorophyll content of fully greened leaves was unaffected by mild water stress (Huffaker et al. 1970). T. urticae feeding injury causes water stress in peppermint leaves through damage to leaf epidermis and cuticle (Chapter II). However, it is not possible at this time to assess the impact of water stress on chlorophyll metabolism in peppermint. Chlorophyll loss may, nonetheless, serve as a useful measure of the degree of cell disruption in spider mite-injured leaves. Sances et al. (1979b) reported that no reduction in extractable chlorophyll was observed in T. urticae-injured strawberry leaves, at injury levels comparable to those of this study. However, in a related study, Sances et al. (1979a) estimated leaf chlorophyll content using a chlorophyll reflectance meter for measuring chlorophyll (in units of absorbance) on each side of strawberry leaflets, separately. In this case, chlorosis
was demonstrated at the highest injury level, but only on leaflet undersides—the side on which mites had been confined. Strawberry is somewhat unusual in that most of the chloroplast-bearing cells are located in the upper palisade layer and therefore are not subject to as much injury when mites are confined to the lower surface (Sances et al. 1979a). These observations support the argument, made above, that general leaf response of chlorophyll metabolism is not responsible for chlorophyll loss in injured leaves, and that at least most of the observed chlorosis can be attributed to mechanical disruption of chloroplast-bearing mesophyll cells.

Figure 18 shows the relationship between photosynthesis and leaf conductance in mite-injured and uninjured peppermint leaves (data from EXP. II). Leaf conductance was calculated as the reciprocal total leaf diffusive resistance (see Chapter II for discussion of leaf resistance network). The regression equations relating photosynthesis and leaf conductance for uninjured and injured leaves were not statistically significantly different (P<.001) with regard either to slope or intercept of the regression lines; the data were therefore combined for Fig. 18. These data show that, at lower leaf conductance (high leaf resistance to gas exchange), photosynthesis is strongly dependent on conductance. At higher conductance (low leaf resistance),
FIGURE 18. Relationship between photosynthesis (Y-axis) and leaf conductance (X-axis) for combined data from EXP. II. 
n=164, 17270.6 + 10582.4 (log X), r² = 0.54. P<.001.
Figure 18

Leaf Conductance (cm·sec⁻¹)

Photosynthesis (10³ DPM)

- UNINJURED
- INJURED

17270.6 + 10582.4 (log X)
however, photosynthesis becomes nearly independent of gas exchange. Aside from local deficiencies in photosynthesis caused by cell disruption at feeding loci, the overriding effect of mite injury on photosynthesis appears to be the decrease in leaf conductance to gas exchange. In Chapter II it was demonstrated that spider mite injury caused a decrease in leaf conductance to gas exchange, probably resulting from stomatal closure in response to mite-induced water stress. In studies that examined the effects of water stress on photosynthesis, it was found that stomatal closure was the dominant factor restricting photosynthesis (Turner et al. 1978). It therefore seems likely that water stress brought on by damage to leaf epidermis and cuticle (Chapter II), accounts in large part for the observed decline in photosynthetic rate associated with mite injury.
Effects of *Tetranychus urticae* Koch Feeding Injury on Physiological Processes in *Mentha piperita* L.

CHAPTER V

Effects on Leaf Carbohydrates and Osmotic Adjustment in Mite-Injured Leaves
ABSTRACT

Studies were conducted to examine the effects of feeding injury by the twospotted spider mite (Tetranychus urticae Koch) on leaf carbohydrates of peppermint, Mentha piperita L. Levels of the predominant soluble leaf carbohydrates (sucrose, raffinose, and stachyose) increased with increasing mite injury. This increase is discussed in terms of an osmotic adjustment mechanism responding to injury-induced leaf water deficits. Levels of leaf starch were essentially unaffected by injury; however, this may have been due to the presence of starch left behind in cell wall debris at feeding sites, causing an overestimation of metabolically available starch.
INTRODUCTION

This is the final chapter in a series describing the physiological responses of peppermint (Mentha piperita L.) to feeding injury by the twospotted spider mite, Tetranychus urticae Koch. The overall objective of these studies has been to identify physiological processes that are important in evaluating spider mite injury, and to characterize injury in terms of these underlying mechanisms. The long-term specific objective is to relate spider mite-induced physiological stress to processes involved in peppermint oil biosynthesis.

Most of the existing literature concerned with effects of spider mites on host plants has dealt with effects on gas exchange—either photosynthesis or transpiration (cf. Boulanger 1958, Hall and Ferree 1975, Sances et al. 1979a, 1979b). A limited number of more recent studies have addressed effects of injury on cell ultrastructure (Summers and Stocking 1972, Tanigoshi and Davis 1978), and plant growth regulators (Avery and Lacey 1968, Stormes 1972). This study is the first in which the effect of injury on non-structural leaf carbohydrates has been examined directly.
Disruption of epidermal and cuticular layers by the probing action of spider mites has been shown to alter gas exchange in affected leaves (Wedding et al. 1958, Atanasov 1971, Sances et al. 1979b, Chapter II, Chapter IV). Injury by T. urticae has been found to produce symptoms of water stress in peppermint leaves (Chapter II). Loomis and Burbott (1976) observed that chronic moisture stress of peppermint induced by high evaporative demand and limited soil water leads to an accumulation of soluble leaf sugars, and suggested that this may serve as an osmotic adaptation to water stress. Osmotic adjustment in response to water deficits involves net accumulation of solutes that effectively lower the osmotic potential of cell sap, causing water to flow into the cell and thereby maintaining turgor pressure at increasingly greater water deficits (Hsiao 1973, Turner et al. 1978). Typically, amino acids and carbohydrates are accumulated during a period of osmotic adjustment (Munns et al. 1979, Ackerson 1981). Data are presented in this paper which suggest that spider mite-induced water stress in peppermint causes an accumulation of soluble leaf carbohydrates, and that this probably reflects an osmotic adjustment mechanism responding to leaf water deficits.

The effects of mite injury on leaf starch accumulation also are presented.
METHODS AND MATERIALS

Two separate series of experiments were conducted, designated EXP. I and EXP. II. The series were similar in design, with minor differences as noted in Chapter II. EXP. I was primarily intended to provide preliminary information on which later experiments could be based. The methods and materials used in the culturing of experimental plants and in the maintenance and culturing of the Tetranychus urticae colony are presented elsewhere (Chapter II).

EXPERIMENTAL DESIGN. Details of the experimental design of EXP. I and EXP. II are presented in Chapter II, along with background information regarding the use of the Mite-Days/cm² injury index. Therefore, only a brief synopsis will be presented here.

One leaf of a pair of peppermint leaves on each plant was infested with virgin female T. urticae at rates of 2, 5, 10, or 20 mites per leaf. The uninfested leaf of the pair served as an intraplant control. Determinations of soluble leaf carbohydrates and leaf starch, as well as other parameters associated with studies reported on in Chapters II and IV, were made on paired leaves sampled at
0, 2, 4, 6, 8, 10, 12, 14, and 16 days (EXP. I), or 3, 6, 9, 11, 13, 15, and 17 days (EXP. II) after infestation. Each day and mite density combination was replicated 4 times for totals of 360 samples in EXP. I and 224 samples in EXP. II.

The Mite-Days/cm² injury index was calculated as shown in Figure 19. All measured parameters were scaled against this index in order to account for variation within treatments in leaf size and mite populations.

Methods of analysis of soluble leaf carbohydrates and leaf starch are given in Chapter III, as well as the methods and materials used for identification of leaf sugars by thin-layer chromatography.
FIGURE 19. Method of calculating the mite-days/cm² injury index used in these studies. "COUNT" refers to the number of living adult female *T. urticae* at the beginning (initial infestation level) of the experiment (N=0) and at each sampling day (N).
Mite-Days/cm² = 

\[ \frac{(\text{COUNT}_0 + \text{COUNT}_N / 2) \cdot N}{\text{AREA OF UNINFESTED LEAF (cm}^2)\} \]

where: 0 & N = DAYS AFTER INFESTATION

Figure 19
RESULTS AND DISCUSSION

The predominant 30% ethanol soluble carbohydrates of peppermint leaves are sucrose, and the galactosyl oligosaccharides raffinose and stachyose. Sucrose accounts for approximately 50% of the sugar present (Chapter III). No attempt was made to distinguish the relative contribution of these three sugars in the results presented below.

Spider mite injury caused a substantial increase in levels of soluble leaf carbohydrates (Figs. 20 and 21). In both series of experiments (EXP. I and EXP. II), but particularly in EXP. II (Fig. 21), there appeared to be an initial decrease in soluble leaf sugar, followed by a rapid rise. The early depression in levels of sugars in injured leaves can probably be attributed to reduced photosynthesis in these same leaves (Chapter IV), possibly reflecting a general slowing down of metabolic processes. Alternative explanations for the observed decline in levels of extractable carbohydrate may relate to enhanced sugar export, or sugar storage. Conversion of soluble carbohydrate to storage forms, however, is not supported by the leaf starch data presented below.
FIGURE 20. Effects of *T. urticae* feeding injury on peppermint leaf soluble carbohydrate (EXP. I). The Y-axis is the ratio of soluble carbohydrate (gm/leaf) of injured to uninjured leaves, expressed as a percent \((\text{injured/uninjured}) \times 100\). The X-axis is mite-days, calculated as mite density \(\times\) duration of feeding \((\text{days})\). Letters refer to the number of replications represented by each point (means): a=27, b=12, c=7, d=8, unlettered points=4. \(n=146; \ 96.6 + 0.28X; r^2 = 0.45; P<.001\).
SOLUBLE CARBOHYDRATE
EXP. 1

Carbohydrate (gm·leaf⁻¹)
(% of uninjured leaves)

Mite-Days

Figure 20
FIGURE 21. Effects of T. urticae feeding injury on peppermint leaf soluble carbohydrates (EXP.II). The Y-axis is the ratio of soluble carbohydrate (gm/leaf) of injured to uninjured leaves, expressed as a percent \(((\text{injured}/\text{uninjured}) \times 100)\). The X-axis is the injury index, calculated as shown in Fig. 19. Each point represents the mean of 4 replicates. \(n=112; 96.6 - 1.02X + 0.214X_i; r^2 = 0.43\).
SOLUBLE CARBOHYDRATE

\[ 96.6 - 1.02X + 0.214X^2 \]

Figure 21
Osmotic adjustment in response to mite-induced water stress may account for the observed increase in levels of soluble leaf carbohydrates in more heavily damaged leaves (Figs. 20 and 21). It is difficult to demonstrate osmotic adjustment per se in any plant experiencing water deficits (Turner et al. 1978b). The difficulty arises in distinguishing passive concentration of solutes due to water loss, from active accumulation attributable to osmotic adjustment (Turner et al. 1978b). The data of Figures 20 and 21 were calculated on the basis of grams of soluble sugar per leaf, not grams per gram leaf weight; comparisons were made between paired leaves on the same plant. It seems reasonable, therefore, to suggest that the data reflect an active accumulation of sugar and not merely an artifact of increasing concentration through water loss. The question remains as to the origin of the additional carbohydrate found in injured leaves.

Turner et al. (1978b) suggested that starch degradation supplies the necessary sugar in osmotically adjusting sunflower and sorghum. Leaf starch may serve this function in peppermint as well, but the data of Figure 22 are not supportive of this hypothesis. Depending on the statistical interpretation, these data indicate that injury has no effect on leaf starch accumulation, or that leaf starch has some tendency to accumulate with injury ($r^2 = 0.04, P \text{ ca.} =$...
FIGURE 22. Effects of T. urticae feeding injury on peppermint leaf starch content (EXP. II). The Y-axis is the ratio of starch (gm/leaf) in injured to uninjured leaves, expressed as a percent \(((\text{injured}/\text{uninjured})\times 100)\). The X-axis is the injury index, calculated as shown in Fig.19. Each point represents the mean of 4 replicates. \(n=112; 105 + 4.7X; r^2 = 0.04; P \text{ ca. } = .030\).
Figure 22: Mite Days × cm⁻² vs. Leaf Starch (% of uninjured leaves)

- STARCH
- Uninjured leaves
There was no evidence, however, for net starch degradation with increasing mite injury, as would be expected if starch served as the source of osmotically active leaf sugar. The interpretation of these data is possibly complicated by the observations of Tanigoshi and Davis (1978) that supposedly metabolically inactive starch was left behind at spider mite feeding sites in apple leaves. This starch would be available for analysis, but unavailable as a source of osmotica for turgor maintenance.

Three opposing effects have been observed in these studies with regard to the effects of *T. urticae* feeding injury on the levels of leaf carbohydrates in peppermint: 1) injury was shown to decrease the net assimilation of carbon (Chapter IV), thus reducing the ultimate supply of carbohydrate; 2) soluble leaf sugars increased in injured leaves, probably in response to water deficits; and 3) leaf starch appeared to be unaffected by injury, at least when measured in the entire leaf. The question of the origin of sugars used as osmotica for maintenance of turgor pressure in injured leaves remains largely unanswered.


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