CHAPTER 4

DISCUSSION

Inhibitory Effect from Methanolic Extracts of E. camaldulensis.

Some scientific papers published on allelopathy suggest that plant growth inhibitors should be extracted only with water in order to simulate the natural release of compounds that might be caused by rain acting on the standing or fallen plant material (Drost & Doll, 1980). Organic solvents permit the extraction of a wide array of organic substances, many of which cannot be leached by rain action. However, in addition to volatilization and leaching, substances may be released from decomposing plant material caused by microbial action. Several investigations have shown that decaying material from inhibitory plants caused growth and germination reduction in sensitive species (Tukey, 1969). Moreover, more thorough extraction may be warranted if the objective is to find unique herbicides (Putnam, 1983). Consequently, the author decided to extract the E. camablulensis material material with methanol which can extract both water-soluble and water-insoluble substances.

1. Germination Inhibition.

Of the three plant parts tesed, bark methanolic extract was the least effective. After the last germination counted, the results indicated that all doses tested were only capable of delaying M. pigra seed germination. On the other hand, germination bioassay of M. pigra seeds with extracts from both green and fallen leaves demonstrated the presence of inhibitory substances at this state. Both

types of the leaf extract exhibited inhibition as well as a delay of M. pigra seed germination. As extract amounts increased, germination reduction generally increased. Investigation showed that when the leaf extract was applied to the seeds of M. pigra, in high doses it completely inhibited germination, while in lower concentrations although germination occurred the resulting seedlings were severely stunted. Germination of O. sativa seeds were less sensitive to the extracts than that of M. pigra seeds. After the last germination counted, the result indicated that all doses tested were only capable of delaying O. sativa seed germination.

2. Seedling Growth Inhibition.

The result indicated that all of the E. camaldulensis material tested appeared to produce a growth inhibitory effect that was concentration dependent. Seedling inhibition was greater with higher amounts of the extracts.

In addition to germination inhibition of M. pigra seeds, methanolic extract from green and fallen leaves also inhibited growth of M. pigra seedlings. While germination was not inhibited, M. pigra growth bioassay with methanolic extract from bark of E. camaldulensis demonstrated the presence of phytotoxin at this state.

All of the extracts tested severely inhibited both hypocotyl and radicle elongation of M. pigra seedlings.

Thus, the phytotoxicity of green leaves methanolic extract stilled remained at a relatively high level in fallen leaves. However, whether the extent of toxicity in fallen leaves was more or less than that of green leaves was not determined. A comparison of the effects

of green and fallen extracts at equal doses (Table 7 and 8 for germination and Table 11 and 12 for seedling growth) showed that at the same dose, fallen leaf extracts were more toxic to the germination and seedling growth of M. pigra. However, this does not indicate an increase of toxic substances in the fallen leaves. Since fallen leaves exist in a drier state there are always more leaf material in fallen leaves than that in green leaves at equal doses.

The extent of inhibition to M. pigra seedling growth by the methanolic extract of green leaves was slightly higher than that of bark.

Compare to the germination, all of the E. camaldulensis material tested exerted their toxic effect mainly upon growth rather than germination. M. pigra seed germination was inhibited only at very high dose (5 g for green leaves and 1 g for fallen leaves). Seeds of O. sativa are even less susceptible than that of M. pigra. After the last germination counted, the results indicated that all doses of green leaves were only capable of delaying O. sativa seed germination so this may not be a major factor in nature. However, Patrick et al. (1964) emphasized that because fallen plant residues are not evenly distributed in the soil, localized pockets of high concentrations of inhibitory substances released from such residues can occur. Therefore, concentrations sufficient to cause germination suppression reducing initial populations might occur in certain soil region. Furthermore, continuous long-term release of these compounds from the decaying material or living E. camaldulensis plants may cause effects not apparent in short-term period.

All of the material tested also inhibited root more than hypocotyl growth. Reduction of root growth would tend to make the affected young plants more susceptible to drought, a significant factor in our hot, dry summer weather. Suppression of root growth make survival less likely and slow growth reduce the vigor of the affected plants.

Isolation of the Inhibitory Factor (s).

Effect of Solvent Extracts of the Green Leaf Methanolic Extract

Fractionation of the methanolic extract showed that the causative factor could be obtained from aqueous and ethyl acetate fraction but not from n-hexane fraction. Most of the activity was concentrated in the aqueous extract. The effect of this extract was nearly as high as that of the original methanolic extract. This suggested that they were in fact polar substances. The ethyl acetate extract of green leaf methanolic extract was less inhibitory than that of bark methanolic extract. This difference may be due to difference in amount and or type of inhibitor. Del Moral et al. (1970) also reported toxicity to Bromus rigidus radicle growth from the aqueous fraction of E. camaldulensis leaf litter. They identified 6 phenolic acids in the aqueous inhibitory fraction, thus, these phenolic acids may be responsible for the observed inhibitory action of aqueous extract from green leaf of E. camaldulensis.

Effect of Solvent Extracts of the Bark Methanolic Extract

It was found that the effect of the inhibitory factor was extracted by both water and ethyl acetate but the n-hexane extract was inactive. The effect of aqueous extract was about two times higher than that of ethyl acetate extract.

The ethyl acetate extract of bark methanolic extract was more inhibitory than that of green leaf methanolic extract. This difference may be due to difference in amount and types of the inhibitors.

Charcoal-Celite Column Chromatography

As expected, most of the inhibitory activities were found in the fractions eluted with polar eluent from charcoal-celite column chromatography. The most polar fraction (50% acetone) gave the highest inhibitory activity. Decreasing polarity of the fractions eluted successively decreased the toxicity of the eluate

Thin-Layer Chromatography

Phenolic compounds have been implicated as some of the common water soluble inhibitors in allelopathy (Whittaker, 1970). Chromatographic analysis of the water/acetone (50/50, v/v) fraction of green leaves of E. camaldulensis revealed two inhibitors one of which (fraction five) had the same color, namely bright white, under short wave UV-light, and the same R_f value as chlorogenic acid. Moreover, later experiment (Table 22, 23, 24 and 25) indicated that synthetic chlorogenic acid showed a pattern of growth inhibition similar to that of fraction five. Thus fraction five was identified as chlorogenic acid. Although the author was unable to identify the other inhibitor

(fraction one) through comparisons of chromatography with well known inhibitors, gallic, ferulic, p-coumaric and caffeic acids were eliminated as possibilities. Fraction one and five had a similar effect on the growth of M. pigra seedlings, both fractions inhibited root growth more than hypocotyl growth. The fifth fraction derived from 1 g of green leaves inhibited M. pigra seedling growth more than that exerted by fraction one derived from the same amount of green leaves. However, fraction five may be less inhibitory than fraction one since the chromatogram (Table 20) indicates that its band size is approximately twice as large as that of fraction one whereas its effect exerting on M. pigra seedling growth is only slightly higher than that of fraction one.

Since the two inhibitors found could be eluted from the charcoal-celite column with water/acetone (50/50, v/v), they must be at least slightly water soluble and could naturally leach out of dead leaves (if they are confined within leaf cells) once the membranes are no longer differentially permeable or of living leaves (if they exist in the outer covering layer of leaves). Moreover, all inhibitors (including these two inhibitors) could be released from the decaying material. Since these toxins are water soluble, during rainy sason, they must be washed deep into the soil and diluted. This mechanism may explain partially why allelopathic phenomena resulted from E. camaldulensis are less easily recognized in certain regions. The results also indicate that the toxins cannot remain in the soil after the donor plants have been removed.

The biochemical effects of chlorogenic acid on metabolism and growth were not investigated. However, chlorogenic acid was

reported to be a strong inhibitor of several enzyme systems including potato phosphorylase and at least one other enzyme requiring pyridoxal phosphate (Sondheimer, 1962).

Effects of 13 Phenolic Compounds on Growth of M. pigra and O. sativa Seedlings.

For the sake of simplicity, the experiments in this section have been limited to an examination of the effects on single compounds. However, under natural conditions, when phenolic compounds have been implicated in allelopathy, complexes of many closely related compounds have been isolated, all of which may be acting in an additive or synergistic manner.

The growth inhibitory activity of the compounds tested seemed to be linked to the presence, position and number of substituents attached to their aromatic rings. Increasing hydroxylation within a series tended to decrease the growth inhibitory activity of the compounds. For example, benzoic acid having no hydroxyl group was the most inhibitory towards M. pigra seedling growth. The precence of additional hydroxyl groups in the aromatic ring of p-coumaric, gentisic, protocatechuic and gallic acids gradually decreased the growth inhibitory activity against M. pigra seedlings (Table 22, 23, 24 and 25). Thus, the higher the polarity, the lower the growth inhibitory activity the phenolic compounds. These results are comparable to those found by Glass (1973, 1974) for inhibition of ion uptake. The presence of one methoxyl group in ferulic acid enhanced its growth inhibitory activity towards M. pigra. The relative posi-

tions of the substituents on the ring are also expected to be significant since protocatechuic acid and gentisic acid is a structurally isomeric pair but gentisic acid displayed more inhibitory activity towards M. pigra seedling growth than the former (Table 22, 23, 24 and 25).

M. pigra seedlings were much more susceptible than O. sativa seedlings to all of the phenolic compounds tested. This indicates that O. sativa may have evolved a mechanism for circumventing the effects of phenolic compounds.

Strangely, increasing in a certain range of concentration of some phenolic compounds tested i.e. benzoic acid, salicylic acid, vanillin, methyl cinnamate and chlorogenic acid, no increase in the inhibitory activity against M. pigra seedling growth was observed. These results were different from those found by Glass (1973, 1974) for inhibiting ion absorption in Hordeum vulgare (barley). The difference may be due to difference in the test species.

Albeit phenolic acids are mainly detoxified via esterification of the carboxyl group of the phenolic acids (Harborne, 1982) chlorogenic acid did not follow the rule since it was more toxic than the corresponding aglycone, caffeic acid.

The present study clearly indicates that benzoic acid cinnamic acid derivatives at concentrations ranging from 10 - 1000 ppm may significantly reduced growth of M. pigra. As mentioned earlier, the occurrence of benzoic and cinnamic acid derivatives in soils is wide-spread and may be in the concentration of approximately 100 ppm in soil solutions. Interestingly, from the viewpoint of allelopathic

effects the concentration range of some phenolic compounds encompassed by this experiment falls well below the concentration of the compounds which have been reported in various soil types. Here comes the question why no apparent inhibition of plant growing in the soils containing the phenolic compounds occur? It must be remembered that phenolic acids would, at least in part, be presented in an adsorbed and bonded form in soils, and that these phenolic acids may completely lose their activities towards plant growth and germination. Thus, the concentration of the free phenolic acids remaining in soil solutions may be too low to exhibit any apparent effects.

On the other hand, the concentration of the free phenolic acids in soil solutions may be high enough to be inhibitory to the plants growing in distilled water containing only cellulase powder like this experiment but inactive under natural conditions. As mentioned in the second chapter, many phenolic acids can decrease the uptake rate of mineral salts being essential to plant growth. In addition to phenolic acids, general soils also contain a large number of mineral salts. The phenolic acids in soils may inhibit only one part of the mineral salts, and the other part may be available for plant utilization.