Introduction

Allelopathy is the inhibitory or stimulatory effect of a plant on another species as a result of the release of chemicals synthesized by itself into the environment. Allelopathy has been a recognized phenomenon for many years and an enormous number of candidate allelochemicals have been identified. In the past few decades allelochemicals with phytotoxic activity have received special attention due to their potential use as environmentally benign herbicides; however, the mode of action of allelochemicals and their practical roles in agricultural ecosystem are not sufficiently understood.

A tropical legume, velvetbean (Mucuna pruriens (L.) DC. ver. Utilis), has been shown to contain a large amount of L-DOPA (L-3,4-dihydroxyphenylalanine) and exudes the compound from the roots. Although L-DOPA is the precursor of some alkaloids, lignin, phenylpropanoids and melanin, excess exogenous L-DOPA suppresses root elongation of several plant species; therefore, it has been considered that the allelopathy of velvetbean can be attributed to L-DOPA. In a survey of the phytotoxic activity of L-DOPA analogues, we found that m-tyrosine (L-3-hydroxyphenylalanine) had comparable activity with L-DOPA. Recently, m-tyrosine was reported as an allelochemical of fine leaf fescue, Festuca rubra. In this article, research on the mechanisms of action and selectivity of L-DOPA and m-tyrosine (Fig. 1) are...
Cytotoxicity of L-DOPA

Hundreds of non-protein amino acids exist in plants and are consumed in animal feed and human food, and some of these amino acids can be toxic to mammals. Among the non-protein amino acids, the effects of L-DOPA on animal cells have been studied intensively. Figure 2 shows the L-DOPA and dopamine metabolism pathway in the brain. L-DOPA is produced via the oxidation of tyrosine by the copper-containing enzyme tyrosinase in the presence of molecular oxygen. Once formed, L-DOPA can be converted into several neurologically important catecholamines, such as the neurotransmitter dopamine by the enzyme DOPA carboxylase, and then the important hormones adrenaline and noradrenaline. Therefore, in Parkinson’s disease, L-DOPA administration is a therapeutic treatment that replenishes the loss of dopamine in dopaminergic neurons. Despite its tremendous beneficial effect, long-term use of L-DOPA may cause well-documented side effects, including oxidative damage to the cells.

L-DOPA is an essential precursor in the biosynthesis of melanin, which is present in many mammalian, insect and plant tissues. In the early stage of melanogenesis, oxidation processes from L-DOPA to DOPA quinone and from dopamine to dopaminquinone are catalyzed by polyphenol oxidase (PPO, EC 1.10.3.1, EC 1.14.18.1, also referred to as tyrosinase) or by autooxidation. Coupled with these oxidation processes, the generation of reactive oxygen species has been reported. The cytotoxicity of L-DOPA in nerve cells is considered to be due to oxidative damage by these reactive oxygen species. In many cases, the cytotoxicity of L-DOPA in nerve cells could be suppressed by antioxidative enzymes or antioxidants.

As a detectable reactive oxygen species, endogenous generation of hydrogen peroxide during the oxidation of L-DOPA and dopamine has been reported in mammalian cells. This is considered to be due to univalent reduction of oxygen molecules. As shown in Fig. 3, the process of hydrogen peroxide generation may include the superoxide anion radical and hydroxyl radical as reactive oxygen intermediates. In the process of L-DOPA oxidation (Fig. 4), its rate is greatly enhanced in the presence of trace concentrations of redox-active transition metal ions such as ferric or cupric ions. Electron oxidation of L-DOPA produces a DOPA semiquinone radical (DOPA-
SQ\textsuperscript{−}), which has an unpaired electron. DOPA-SQ\textsuperscript{−} readily undergoes further electron oxidation to DOPA quinone. This process can occur by electron transfer to metal ions with suitable redox properties, molecular oxygen and other electron acceptors, such as peroxides, or via disproportionation of two DOPA-SQ\textsuperscript{−} molecules. Several studies have also pointed out the involvement of quinone compounds produced from dopamine in the induction of cytotoxicity.\textsuperscript{21,22) DOPA quinone and dopaminquinone are then transformed into brown melanin-like compounds \textit{via} several steps, including oxidation and polymerization.\textsuperscript{23) In plant cells, L-DOPA is also metabolized to several catecholamines, phenylpropanoids, and melanin\textsuperscript{5,}; however, the phytotoxicity of dopamine is much less than that of L-DOPA\textsuperscript{5) although L-DOPA was also metabolized to dopamine in plants.\textsuperscript{7,24,25) Furthermore, Nishihara \textit{et al.} showed that perennial ryegrass, one of the most tolerant species to L-DOPA among gramineous plants, metabolized L-DOPA to dopamine.\textsuperscript{6) These results suggested that the metabolic pathway not from L-DOPA to dopamine but that to DOPA quinone is mainly involved in L-DOPA action in plants.

**Mechanisms of Phytotoxicity and Selectivity of L-DOPA**

Many non-protein aromatic amino acids have been found in plants (Table 1); however, their phytotoxic activity has not been reported, except for L-DOPA and m-tyrosine. The phytotoxicity of L-DOPA was first reported by Fujii \textit{et al.}\textsuperscript{3) by demonstrating radicle growth inhibition of lettuce by an extract from velvet bean leaves. From the determination of L-DOPA content in the extract, they concluded that the phytotoxic activity was due to L-DOPA. Early research on L-DOPA action showed that L-DOPA suppressed the growth of roots more significantly than shoots and the inhibitory effect was selective among plant species.\textsuperscript{5) This group pursued L-DOPA work and Nakajima \textit{et al.}\textsuperscript{26} reported that L-DOPA-treated cucumber (\textit{Cucumis sativus} L.) accumulated large mounts of phenylalanine and tyrosine in the roots. The authors assumed that the increase of these amino acids was due to the metabolism of L-DOPA and could be one of the detoxification mechanisms of some plant species. Furthermore, Nishihara \textit{et al.}\textsuperscript{6) showed that the level of growth inhibition by L-DOPA was dependent on species and suggested the involvement of the metabolic activity of L-DOPA in differential sensitivity among plant species. Germinating perennial ryegrass (\textit{Lolium perenne} L.) was tolerant to L-DOPA and this was considered to be due to its metabolism of L-DOPA to dopamine. Thereafter, they demonstrated the actual exudation of L-DOPA from velvet
however, the action mechanism of L-DOPA in plants, including selective phytotoxicity, has not been clarified.

We examined the phytotoxic effect of L-DOPA on 32 species and found that the compound had selective toxicity among the tested species. The sensitivity of plant species was not related with their morphology (monocot or dicot) or type of carbon fixation (C3 or C4). From these plants we selected barnyardgrass (*Echinochloa crus-galli* L.) and lettuce (*Lactuca sativa* L. cv. Great Lakes 366) as tolerant and susceptible species, respectively, for further study of the selectivity mechanism. When the plants were grown in agar medium containing 0.1 mM L-DOPA, the root lengths of barnyardgrass and lettuce were approximately 80% and 20% of the untreated control, respectively, 5 days after treatment (DAT). Based on GR50 values determined 5 DAT, barnyardgrass was 77-fold more tolerant than lettuce. We then compared the absorption, translocation and metabolism of 14C-L-DOPA in barnyard-

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grass and lettuce. The study showed that absorption or translocation was not a factor of selectivity between the species but the higher detoxification activity of L-DOP A in barnyardgrass was partially involved in tolerance.

In the study of the mode of action of L-DOP A, we used suspension-cultured carrot cells and the generation of reactive oxygen species from the melanin synthesis pathway and their involvement in phytotoxicity were investigated. When the cells were treated with 1 mM L-DOP A, growth was inhibited at 2 days after treatment and the cells were dark, suggesting melanin formation. In carrot cells, melanin content increased continuously for 6 days and was dependent on L-DOP A concentration. Lipid peroxide formation determined as thiobarbituric acid reactive substances (TBARS) was also greater in L-DOP A-treated cells than in the non-treated control, especially 2 days after treatment.

We also determined the effect of antioxidants on the growth of L-DOP A-treated cells. Interestingly, exogenously applied ascorbate completely recovered cell growth at 4 DAT. Recovery by α-tocopherol was imperfect but was more effective at an earlier stage of L-DOP A exposure. Furthermore, these antioxidants completely suppressed melanin accumulation and lipid peroxide formation. This series of studies strongly suggested that L-DOP A suppressed cell growth by generating reactive oxygen species from the melanin synthesis pathway.

These responses in carrot cells were confirmed in intact plants. In barnyardgrass and lettuce, melanin formation correlated well with the growth inhibition of their roots. Levels of TBARS were much higher in L-DOP A-treated lettuce than in the non-treated control but did not change in barnyardgrass. In lettuce, root growth was more strongly suppressed under greater accumulation of TBARS; however, the root length of lettuce seedlings was rescued up to 80% of the treated control when 5×10^-8 M ascorbic acid was treated with L-DOP A. Ascorbic acid was more effective than α-tocopherol in suppressing phytotoxicity. The antioxidant effect on the formation of TBARS and melanin in the roots of L-DOP A-treated lettuce was not clear compared with in carrot cells; however, TBARS content decreased with antioxidant treatment. Therefore, it was considered that the reactive oxygen species generated from the melanogenesis pathway, especially from the metabolic process from L-DOP A to DOP A quinone, were involved in the phytotoxic action of L-DOP A. This toxic oxygen may cause oxidative damage and eventually growth inhibition and cell death (Fig. 5).

As mentioned above, enzyme polyphenol oxidase (PPO) is involved in the enzymatic conversion of L-DOP A to DOP A quinone. It is also known as a key enzyme in the regulation of the melanogenesis pathway. Determination of polyphenol oxidase activity in roots revealed that lettuce had much higher activity than barnyard grass, suggesting that the melanin synthesis pathway is more active in lettuce. This may lead to a greater amount of reactive oxygen and melanin generation. Thus, the difference in PPO activity between the two species is likely to be one of the factors conferring species-selective phytotoxicity. Furthermore, we found that ascorbic acid suppressed PPO activity in lettuce. In contrast with ascorbic acid, α-tocopherol did not affect PPO activity.

The possible mechanisms of phytotoxic action and selectivity of L-DOP A are summarized in Fig. 6. Phytotoxicity of L-DOP A

![Fig. 5. Phytotoxic mechanism of L-DOP A in plants.](image-url)
DOPA is due to oxidative damage caused by reactive oxygen species generated from the melanin synthesis pathway. Selectivity among species might be achieved by differential activity of PPO. Plant species with high PPO activity introduce more L-DOPA to the melanin pathway and generate a greater amount of toxic reactive oxygen species. Other species with lower PPO activity may metabolize L-DOPA to other nontoxic metabolites. In the action of antioxidants, ascorbic acid reduced melanin formation and ROS generation by suppressing PPO activity. The protective action of lipid peroxidation by α-tocopherol might be due to quenching free radical and reactive oxygen species in lipophilic layers.

**Determination of Reactive Oxygen Species Involved in L-DOPA Action**

The mechanism of the generation of reactive oxygen species by L-DOPA has also been studied. Employing specific and sensitive electrochemical methods, the generation of hydrogen peroxide (H₂O₂) and other reactive intermediate of oxygen, possibly superoxide anion (O₂⁻), during the oxidation of L-DOPA to DOPA quinone but not during the hydroxylation of tyrosine to L-DOPA was demonstrated in vitro. In another study, the generation of hydroxyl radical by autooxidation of L-DOPA was well demonstrated by electron spin resonance (ESR) spectrometry. In the brains of Parkinson’s disease patients, dopamine markedly reduced the viability of neuroblastoma cells. In these cells, the generation of dopamine-semiquinone was detected by ESR but semiquinone generation and cytotoxicity were both prevented by superoxide dismutase or glutathione, suggesting the involvement of superoxide radical in the toxic action. In insect cells with high melanogenesis activity, the formation of DOPA semiquinone and its involvement in cytotoxic reactions through reactive oxygen generation have been well demonstrated. In our ESR study, radical signals were detected when polyphenol oxidase extracted from lettuce or obtained commercially was added to L-DOPA solution but no signal from the mixture of L-DOPA and barnyardgrass extract. The radical signal disappeared with the addition of ascorbic acid. Considering L-DOPA metabolism in plants, the involvement of radical species, presumably DOPA-semiquinone, in the toxic action is strongly suggested.

**Phytotoxic Activity and Action Mechanism of m-Tyrosine**

From a survey of the phytotoxic activity of the structural analogues of L-DOPA, we found that m-tyrosine had strong growth suppression activity in lettuce roots and shoots. Furthermore, m-tyrosine suppressed barnyardgrass growth, which was tolerant to L-DOPA. Similarly to L-DOPA, m-tyrosine induced lipid peroxide formation in lettuce roots, suggesting that m-tyrosine also caused oxidative damage; however, unlike L-DOPA, this oxidative damage was not rescued by the antioxidants ascorbic acid or α-tocopherol, but by an amino acid, phenylalanine. In the comparison of melanin formation from L-DOPA and m-tyrosine, less melanin was synthesized from m-tyrosine. From the results we concluded that the mechanisms of phytotoxic action and selectivity of m-tyrosine are different with those of L-DOPA. Two years after our first report, Bertin et al. found m-tyrosine to be the allelochemical
in fine fescue grass (Festuca rubra) responsible for displacing neighboring plants. They previously reported that root exudates of some fescue species were phytotoxic but the active substance was not identified. From the bioassay of m-tyrosine showing growth inhibition of a wide range of plant species, they proposed that the release of this non-protein aromatic amino acid interfered with the root development of competing plants. They also showed that the phytotoxicity of m-tyrosine on Arabidopsis was rescued by amino acids, especially by phenylalanine and methionine.

It has been known for decades that certain structural analogs of protein amino acids can escape detection by the cellular machinery of protein synthesis and become misincorporated into the growing polypeptide chain proteins. The incorporation of amino acid analogues was well-established in early studies of protein synthesis. As a mechanism of cytotoxicity, misincorporation of m-tyrosine in place of phenylalanine into cellular protein was suggested in bacteria and cultured Chinese-hamster ovary cells. Plant amino acids having anti-microbial and insecticidal activities revealed that one mechanism of toxicity was the incorporation of non-protein amino acids into the cellular protein of predators; however, the biosynthetic incorporation of m-tyrosine into plant systems has not been well studied. Mung bean phenylalanine tRNA synthase accepts m-tyrosine with 25% efficiency of phenylalanine, suggesting that m-tyrosine might also be misincorporated into plant proteins; a small amount of m-tyrosine was detected from the root protein of m-tyrosine-treated Arabidopsis seedlings. In a series of laboratory assays performed in field soil, Bertin et al. showed that m-tyrosine inhibited the root growth of lettuce seedlings but growth inhibition was strongly reduced when significant amounts of activated carbon were added to the soil medium. They suggested the potential of m-tyrosine for development as a soil-applied herbicide; however, our recent results suggested that m-tyrosine is not incorporated into the proteins of rice roots, which are very sensitive to m-tyrosine. Much of the evidence also supports the view that the misincorporation of modified non-protein amino acids in vivo would not normally occur to any significant extent. A more recent study using Arabidopsis mutants, which are resistant to m-tyrosine, showed the huge accumulation of free phenylalanine in leaves, suggesting the involvement of the metabolic response in the resistance to m-tyrosine; however, the mechanism of phytotoxicity of m-tyrosine is not yet fully understood.

Conclusion and Perspectives

Several non-protein aromatic amino acids, such as l-DOPA and m-tyrosine, have been implicated in allelopathy. Our study strongly suggests that they have different modes of action, although their chemical structures are very similar. The susceptibility of plant species to these chemicals is also different and m-tyrosine is more broadly phytotoxic. The action and selectivity mechanisms of l-DOPA in plants have been clarified as described but those of m-tyrosine are still unknown; therefore, the precise mechanisms of m-tyrosine should be investigated. Another assignment is to clarify the mechanism that the producing plant uses to avoid the effects of allelopathic compounds. As possible mechanisms avoiding autotoxicity: (1) rapid secretion to prevent accumulation of the compounds in the cell, (2) sequestration into intracellular or intercellular locations where they can do little or no harm, (3) metabolic detoxification to a less toxic compound, and (4) resistance at molecular target sites, have been considered. Therefore, it should be determined how Mucuna pruriens and Festuca rubra protect themselves from the toxicity of l-DOPA and m-tyrosine, respectively. l-DOPA is metabolized to dopamine in Mucuna pruriens leaves but there is obviously a paucity of information on the mechanism at present. For current and future agricultural production, new herbicides with a novel structure and new site of action are needed. These herbicides should also be environmentally more friendly and beneficial to crop production; therefore, allelochemicals should be further isolated and identified from allelopathic plants to develop herbicides with new modes of action.

References