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Distribution of Canavanine and Free Amino Acids in Legumes, *Robinia pseudo-acacia*, *Wistaria floribunda*, and *Canavalia lineata*

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콩과식물인 아까시나무(*Robinia pseudo-acacia*), 등나무(*Wistaria floribunda*) 및 해녀콩(*Canavalia lineata*)에서 canavanine과 유리아미노산의 분포

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ABSTRACT

The constituents and proportions of non-protein free amino acids including canavanine were measured in roots and leaves of legumes, *Robinia pseudo-acacia* L., *Wistaria floribunda* L., and *Canavalia lineata* L. by using high performance liquid chromatography during dormant and fertilizing seasons. In all the three plant species, asparagine was the most abundant amino acid occurring 30% of total free amino acids, and canavanine was the second most abundant amino acid contributing 10% of total free amino acids throughout dormant and fertilizing seasons. In dormant season, roots contained 2 to 3 folds of free amino acids including canavanine and asparagine compared to those in fertilizing season. When proportions of asparagine and canavanine to total free amino acids in various parts of *C. lineata* were examined in fertilizinng season, the level of asparagine was the highest in roots while that of canavanine was in seeds. On the basis of these results, it is assumed that canavanine appears and functions as a nitrogen-storing compound in roots and leaves throughout the whole life cycle of the investigated plants.

Key words: Canavanine, Free amino acids, Dormant season, Fertilizing season, *Robinia pseudo-acacia*, *Wistaria floribunda*, *Canavalia lineata*

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INTRODUCTION

Plants have the ecological ability of defense against insects, fungi, and grazing animals to overcome to be over-eaten. One of the plant's defense is based on chemical factor, which is provided by the nitrogen-based plant toxins. Of these toxins, the simplest in structure are the non-protein amino acids.

L-Canavanine [2-amino 4-(guanidinooxy)butyric acid] is a non-protein amino acid and a structural analogue of L-arginine (Kitagawa and Tomiyama 1929, Rosenthal 1988). The apparent restriction of canavanine to Leguminosae and its occurrence in 1200 species representing 240 genera have proved of real value in delineating phylogenic relationships in the Leguminosae (Bell 1971). The studies with canavanine containing species indicate that canavanine stored in seeds represents 4 to 10% of the seed dry weight (Rosenthal 1982).

The toxicity of canavanine has been demonstrated in a variety of organisms including herbivorous insects (Rosenthal 1977, 1982). Because of similarity in structure between canavanine and arginine, canavanine is a substrate for arginyl tRNA synthetase resulting in structurally aberrant proteins with impairing biochemical function (Allende and Allende 1964, Rosenthal 1988).

On the other hand, due to the high proportion of nitrogen to carbon in canavanine molecule, canavanine has been assumed to play a role as a nitrogen-storing house in plants (Park and Kwon 1990, Rosenthal *et al.* 1988, Yu and Kwon 1992). Among the legume plants, *Robinia pseudo-acacia* L., *Wistaria floribunda* L., and *Canavalia lineata* L. has been known as canavanine-containing plants in their seeds (Bell 1971). Especially *C. lineata* contains canavanine accounting for more than 10% dry mass of its seed (Kwon *et al.* 1986). However, the explanation of canavanine as a nitrogen storage compound has not been fairly revealed in vegetative organs of plants.

In the present study, we have estimated the proportions of non-protein free amino acids including canavanine in vegetative organs, such as roots and leaves, in dormant and fertilizing seasons. We have also attempted to show the possibility that canavanine plays a role as a nitrogen-storing compound in vegetative organs as well as in seeds of plants.

MATERIALS AND METHODS

Plant Materials

The roots and leaves of *Robinia pseudo-acacia* L. and *Wistaria floribunda* L. were collected at Kwan-Ak Mountain, and those of *Canavalia lineata* L. were sampled at Jeju Island, Korea, in dormant and fertilizing seasons. Samples were stored at −80℃ before measuring free amino acids.

Extraction and measurement of free amino acids

Frozen plant materials were ground with 70% of ethanol and centrifuged at 12000 g for 10 min. Fifty percent of ethyl acetate was added to the resulting supernatant solution and then soluble fraction was taken for determination of free amino acids by using colorimetric assay with ninhydrin (Plummer 1978). To measure the proportions of free amino acids with HPLC (high performance liquid chromatography) the above extracts were further purified with Dowex-50-X column according to Park and Kwon (1990).

Determination of constituents and proportions of free amino acids with HPLC

The HPLC system consisted of a Beckman 110 B solvent delivery module, a Beckman 163 variable wavelength UV-detector, a Beckman ultrasphere ODS RP C_{18} precolumn (5 μm, 4×4.5 mm) and Waters Nova Pak ODS RP C_{18} column (4 μm, 3.9×150 mm). The coupling of amino acids with PITC (phenylisothiocyanate) was accomplished according to Heinrikson and Meredith (1984). The solvent systems were based upon aqueous ammonium acetate with acetonitrile and methanol (Yu and Kwon 1992). The flow rate was 1 ml/min and the PITC derivatized amino acids were detected at 254 nm. Proportions of amino acids were obtained from proportion of peak area of each amino acid to total peak area.

RESULTS AND DISCUSSION

Constituents of free amino acids were measured with HPLC in roots and leaves of three different plant species, already known as canavanine-containing legumes in their seeds, in dormant and fertilizing seasons (Table 1).

In the present study, dormant season implies winter when metabolic activity is lowest, and fertilizing season is when the plants are flowering and producing seeds. Samples of dormant season were collected in February when leaves had not come out yet. Thus free amino acids in leaves were not measured in dormant season. Leaves and roots in fertilizing season were collected in May for *R. pseudo-acacia* and *W. floribunda*, and in August for *C. lineata*.

Throughout all the three plant species, the most abundant free amino acid was asparagine occurring about 30% of total free amino acids. While the propotion of arginine was less than 5% except *R. pseudo-acacia* roots in fertilizing season, level of canavanine, which is a guanidinooxy analogue of arginine and a toxic allelochemical, was around 10% of the total free amino acids and represented the second most abundant amino acid. Besides the six amino acids, such as aspartic acid, glutamic acid, asparagine, glutamine, canavanine, and arginine, the other compounds included a large amount of an unidentified compound contributed 30 to 50% of the total free amino acids. It was convincing that the unidentified compound was not a free amino acid, since the retention time of its peak on HPLC was not correlated with retention times of standard amino acids. However, we have not further investigated to identify this compound. The sum of proportions of other free amino

Table 1. Constituents and proportions of free amino acids in roots and leaves of *Robinia pseudo-acacia* L., *Wistaria floribunda* L., and *Canavalia lineata* L. in dormant and fertilizing seasons

Free amino acids	Dormant season[a]	Fertilizing season[b]	
	Roots	Roots	Leaves
	% total free amino acids		
Robinia pseudo-acacia L.			
ASP	2.7	2.7	4.5
GLU	0.3	1.2	0.3
ASN	25.9	23.7	16.4
GLN	0.04	2.7	4.2
CAN	9.1	12.7	10.9
ARG	5.1	6.8	2.4
Others[c]	56.9	50.2	61.3
Wistaria floribunda L.			
ASP	1.7	2.3	0
GLU	0.1	3.7	0.4
ASN	23.0	20.7	18.7
GLN	0.3	5.4	4.7
CAN	9.3	11.7	7.5
ARG	0	0.08	2.2
Others[c]	65.6	56.1	66.5
Canavalia lineata L.			
ASP	2.5	4.5	4.4
GLU	3.2	3.0	1.1
ASN	34.0	51.9	31.5
GLN	0.6	0.8	2.1
CAN	10.7	6.9	8.1
ARG	1.4	2.0	2.2
Others[c]	47.6	30.9	50.6

[a]: February

[b]: May for *Robinia pseudo-acacia* and *Wistaria floribunda*; August for *Canavalia lineata*.

[c]: α− amino groups except ASP (aspartic acid), GLU (glutamic acid), ASN (asparagine), GLN (glutamine), CAN (canavanine), and ARG (arginine).

acids except the six amino acids we measured and the unknown compound was less than 10% of the total.

The ratio of aspartic acid to asparagine was less than 0.3 in roots of the three plant species in both seasons. Also, the ratio of glutamic acid to glutamine was less than 1.0. When nitrogen nutrient is sufficient, the ratios of asparagine to aspartic acid and glutamine to glutamic acid usually increase higher (Rufty *et al.* 1982). This is known to be caused by the incorporation of surplus nitrogen to aspartic acid and glutamic acid, which results in an increase of asparagine and glutamine (Pate and Atkins 1983). Thus, high levels of amides, such as asparagine and glutamine, compared to aspartic acid and glutamic acid indicated nitrogen nutrient was not a limiting factor in growing environments of the three plant species. Since all the three plants were legumes which were able to fix

Table 2. Contents of free amino acids in roots and leaves of *Robinia pseudo-acacia* L., *Wistaria floribunda* L., and *Canavalia lineata* L. in dormant and fertilizing seasons. Values are mean ± a standard error

Species	Dormant season[a]	Fertilizing season[b]	
	Roots	Roots	Leaves
	Free amino acids (μmol /g FW)		
Robinia pseudo-acacia L.	26±2.3	7.7±2.5	7.7±0.5
Wistaria floribunda L.	22.1±0.9	12.9±1.0	26.3±2.7
Canavalia lineata L.	66.3±7.4	39.8±0.4	51.4±0.6

[a]: February

[b]: May for *Robinia pseudo-acacia* and *Wistaria floribunda*; August for *Canavalia lineata*.

atmospheric nitrogen to organic nitrogen, there was no nitrogen deficiency throughout their life cycle.

Surprisingly, canavanine was a prominent constituent of free amino acids throughout their life cycle. We have tried to measure the amount of canavanine with colorimetric assay developed by Rosenthal (1976). The colorimetric assay, however, was interfered by the secondary metabolites. Therefore free amino acid contents were measured with colorimetric assay using ninhydrin to quantify canavanine indirectly in the plants by comparing the proportion of canavanine to total free amino acids (Table 2). In dormant season roots contained 2 to 3 times more free amino acids than those in fertilizing season. Considering the same proportion of canavanine to total free amino acids in both seasons (Table 1), roots in dormant season contained 2 to 3 folds of canavanine than that in fertilzing season. This result shows that roots function as a storage organ of nutrients including free amino acids as a nitrogen nutrient during winter for preparing sprouting in the following spring. Furthermore the increase in free amino acids might be due to cold hardiness, which resulted in the increase in solute concentrations in the plant cell. Asparagine and canavanine were the major compounds of amino-nitrogen among free amino acids in winter season. In fertilizing season leaves contained higher level of free amino acids compared to roots, which might result from the higher metabolic activity in leaves.

In the middle of seed formation during fertilizing season, proportions of canavanine and asparagine were measured from lower to upper parts of *C. lineata* (Fig. 1). Seeds and seed pods showed high level of canavanine content which was 20 to 40% of total free amino acids. In contrast to seeds and seed pods, roots, leaves, and stems contained relatively low level of canavanine content. On the other hand, asparagine level was the highest in roots, which was the lowest part of plants and the place fixing nitrogen, and then decreased by 40% in the upper parts of plants. We examined the distribution of canavanine in flowers, and found there was no canavanine in flowers.

It is speculated that nitrogen is actively allocated to asparagine in roots while canavanine in seeds and seed pods. It has been suggested that seed pod synthesized canavanine temporarily; machinery of canavanine synthesis that is supposed to be located

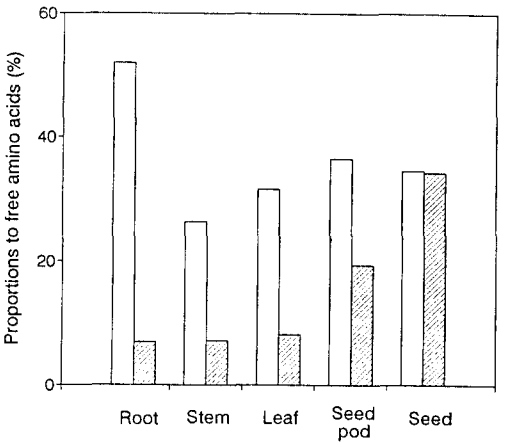

Fig. 1. Proportions of asparagine and canavanine to free amino acids at various parts of *C. lineata* in fertilizing season. Open bar, asparagine; Hatched bar, canavanine.

in seed pod is switched on during fertilization period (Rosenthal 1971). Thus the presence of canavanine in seeds provides a general defense against insect attack (Rosenthal 1977, 1982).

If machinery of canavanine synthesis is switched on during a certain short period and in a specific organ, such as seed pod, canavanine should not appear in vegetative organs before or after canavanine synthesis. While the ecological function of canavanine in legume seeds is fairly clear, the role of canavanine as a nitrogen storing compound in vegetative organs of legume other than legume seeds has not been entirely convincing. However, we have detected a significant level of canavanine throughout the whole life cycle of these perennial plants.

It has been revealed that much of the seed canavanine was exhausted in the cotyledons before cotyledons wrinkle and abscise (Rosenthal and Rodes 1984, Kwon *et al.* 1986). Canavanine depletion as a function of growth and high nitrogen content which is shown by high ratio of nitrogen to carbon suggests that the major function of canavanine is a nitrogen-storing house in plants containing canavanine (Rosenthal 1977, Park and Kwon 1990). Nitrogen utilization from canavanine in canavanine-containing legume commences with the enzyme arginase, which converts canavanine to canaline and urea. The second step in the process of canavanine catabolism involves the enzyme urease, which degrades urea to carbon dioxide and ammonia (Rosenthal 1971, Kwon *et al.* 1986). Canavanine-containing legume produces an arginase that is much more reactive with canavanine as a substrate than arginase of the soybean, a canavanine-free legume (Downum *et al.* 1983, Kavanaugh *et al.* 1990).

In contrast to canavanine catabolism, canavanine synthesis occurs via enzymes of Krebs-Henseleit cycle including arginase (Roubelakis and Kliewer 1978). All the enzymes needed for canavanine synthesis are located in leaves (Rosenthal 1972). Therefore there is a possibility that canavanine is synthesized in leaves as well as in seed pods. Also we assume canavanine is synthesized throughout the whole life cycle rather than only during fertilizing season, which results in consistency that major function of canavanine is a nitrogen-storing house as well as an allelochemical defense against insects.

적 요

콩과식물인 아까시나무(*Robinia pseudo-acacia* L.), 등나무 (*Wistaria floribunda* L.) 및 해녀콩 (*Canavalia lineata* L.)의 잎과 뿌리에서 canavanine을 포함하는 비단백질성 유리 아미노산 조성과 함량비를 high performance liquid chromatography를 이용하여 휴면시기와 생식시기별로 측정하였다. 조사대상 식물체들의 휴면시기와 생식시기 전과정에서 asparagine의 함량비가 전체 유리 아미노산의 30%로 가장 높았으며 canavanine의 함량비는 전 유리아미노산의 10%로 두번째로 높았다. 휴면시기와 생식시기의 뿌리에서 asparagine과 canavanine을 포함한 유리아미노산의 함량을 조사한 결과 휴면시기의 뿌리에서 2~3 배 정도 높게 나타났다. 생식시기의 해녀콩에서 식물체 부위에 따른 유리아미노산의 분포를 조사한 결과 뿌리에서는 asparagine이, 종자에서는 canavanine이 가장 높은 함량비로 나타났다. 이러한 결과들로 부터 canavanine은 콩과식물인 등나무, 아카시아, 해녀콩의 전 생육기간에 걸쳐 분포하며 일종의 질소저장화합물로서 존재하는 것으로 추정되었다.

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