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Soybean as a Nitrogen Supplier

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1. Introduction

Soybean has been cultivated all over the world since ancient times for its high protein and lipid content. It is one of the most important agricultural products in the world and its global production is more than 220 million tons per year [1]. Vegetable oil production from soybean is the highest among plant oils (30%) [2].

Soybean is used directly as food in Japan and several Asian countries. Recently, soybean protein was recognized as both healthy and tasty and is used in food such as Tofu and soy sauce. Soybean-meal, which remains after extraction of the vegetable oil, contains about 50% protein with well balanced amino acids. Therefore, soybean-meal is often re-utilized as animal foodstuff.

Soybean waste was utilized as an organic fertilizer prior to the 1940s [3-6]. However, a chemical fertilizer took the place of the organic fertilizer because it produced faster results. Organic fertilizers are now gradually being used again for increased food production safety and the protection of the environment.

Soybean cultivation is well known for improving soil fertility [3, 7, 8]. Root-nodules are formed by the soybean plant, and atmospheric N_2 is fixed by the nitrogen fixing bacteria in the root-nodule [9]. N_2 is converted to NH_4^+ by nitrogenase from these nitrogen fixing bacteria, and this NH_4^+ is supplied to the soil environment.

Recently, investigations into the utilization of proteins from soybean waste have been carried out for the development of high quality foods. Protein fractions, such as soy protein isolates and whey protein are industrially produced, and these fractions are used as additives for the improvement of food nutrition [10]. Moreover, several soybean proteins and peptides have been

purified and utilized as medicines for hypotension, rheumatism, and cholesterol control [11-13]. The bioactive peptides of soybean protein have also been investigated [5, 6].

This chapter explains how soybean cultivation and soybean protein are nitrogen suppliers and describes the production of novel bioactive peptides from soybean and legumes.

2. Nitrogen supply by soybean cultivation

2.1. Nitrogen fixing bacteria

N₂ is fixed by nitrogen fixing bacteria in the soil environment [14-17]. These bacteria convert N₂ to NH₄⁺. The biological reduction of atmospheric N₂ to NH₄⁺ (nitrogen fixation) provides about 65% of the biosphere's available nitrogen [18].

As long ago as 1890, a nitrogen fixing bacteria was isolated from a root nodule and identified as *Rhizobium leguminosarum* [19, 20]. Shortly after this, *Clostridium pasteurianum* and *Azotobacter* sp. were also isolated as nitrogen fixing bacteria in the soil environment [21-23]. Now, more than 100 genera have been isolated and identified as nitrogen fixing bacteria. Among them, genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Frankia* lead to the formation of root-nodules in legumes [16].

Nitrogenase (EC 1.18.6.1) from nitrogen fixing bacteria catalyzes N₂ to NH₄⁺ ($\text{N}_2 + 8\text{H}_2 + 8\text{e}^- + 16\text{ATP} + 16\text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{ADP} + 16\text{Pi}$). NH₄⁺ is further converted to NO₂⁻ and NO₃⁻ by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB).



Figure 1. Soybean root nodule

2.2. Relationship between nitrogen fixing bacteria and soybean cultivation

The roots of soybean secrete flavonoids and enhance the growth of nitrogen fixing bacteria around the root [24]. The nitrogen fixing bacteria infect the soybean root, and the root-nodule is formed. Bacteroids in the root-nodule fix and provide nitrogen from the air [25]. *Bradyrhizobium japonicum*, *B. elkanii*, *B. lianigense*, and *Sinorhizobium fredii* have been identified as the root-nodule forming bacteria in soybean cultivation [16, 26, 27].

The change in soil microbial diversity after soybean cultivation has been analyzed by PCR-DGGE. Root-nodules were shown to be formed and specific bacteria were increased during cultivation (Figures 1 and 2) but not the total number of bacteria in the soil. Soybean cultivation caused nitrogen accumulation in the soil environment.

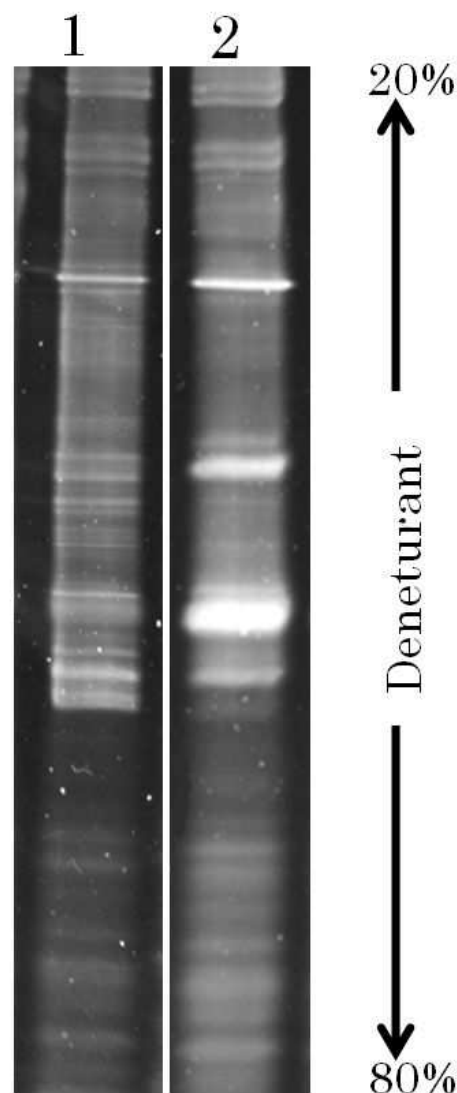


Figure 2. PCR-DGGE profiles of soybean cultivated soil, 1: Before cultivation, 2: after cultivation.

3. Enhancement of nitrogen circulation by soybean cultivation and soybean protein

3.1. Evaluation of nitrogen circulation in soil environment

The nitrogen cycle is illustrated in Figure 3. Organic forms of nitrogen such as protein are degraded to peptides and amino acids by soil microorganisms, and these peptides and amino acids are then converted to NH_4^+ . Subsequently, NH_4^+ is further converted to NO_2^- and NO_3^- (nitrification). NO_2^- is denitrified to N_2 by denitrifying bacteria and this N_2 is converted to NH_4^+ by the nitrogen fixing bacteria, and NH_4^+ is accumulated in the soil environment again.

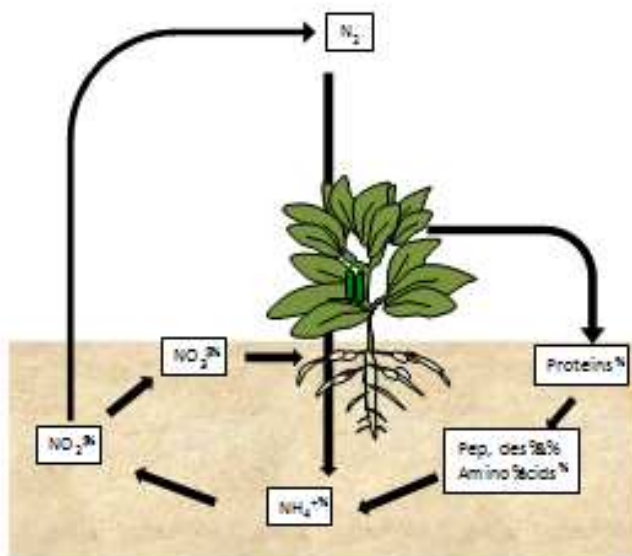
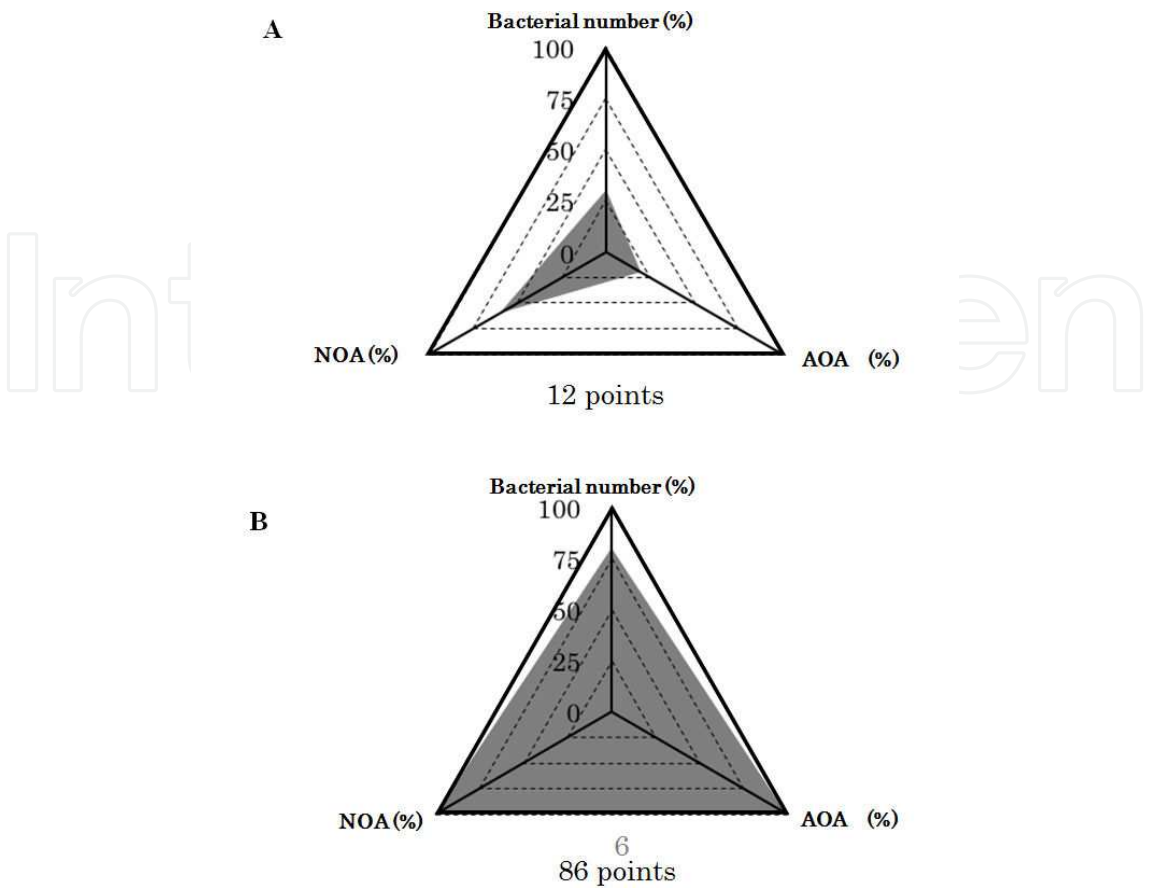


Figure 3. The soil nitrogen cycle

The nitrification process is the rate limiting step in the nitrogen cycle [28]. To further investigate the soil nitrogen cycle, a new method for the evaluation of nitrogen circulation activity was constructed based on bacterial number, ammonium oxidizing activity (AOA), and nitrite oxidizing activity (NOA) (Figure 4) [29]. These three indices were used to construct a radar chart of nitrogen circulation in the soil. The area of the radar chart was calculated, and then the value was treated as a nitrogen circulation activity (0–100 points).

3.2. Enhancement of nitrogen circulation

A database of nitrogen circulation activity was constructed using 155 agricultural soils (Figure 5). The nitrogen circulation activity of agricultural soil ranges from 0 to 99.6 points with an average of 26 points.



A: Un-fertile soil, B: fertile soil.

Figure 4. Values of nitrogen circulation activity in soil environments

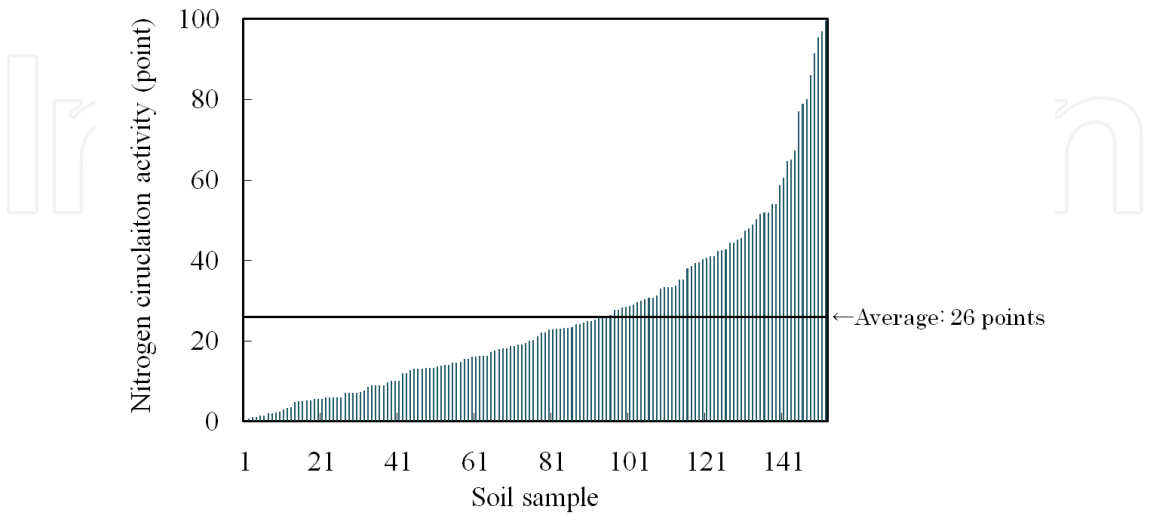


Figure 5. Database of nitrogen circulation activity in 155 agricultural soils

Soybean cultivation leads to nitrogen accumulation in the soil environment, and therefore nitrogen circulation activity should be enhanced by soybean cultivation. This enhancement was further analyzed (Figure 6) and activity was shown to be enhanced 26 to 95 points after soybean cultivation.

Soybean waste is also rich in nitrogen (Table 1), and is often used as an organic fertilizer. Soil nitrogen is increased by using soybean waste as fertilizer, and consequently nitrogen circulation is increased. Soybean waste is also rich in carbon (C/N values; 5.1), and therefore soil bacteria and bacterial activity may also be increased by the addition of soybean waste.

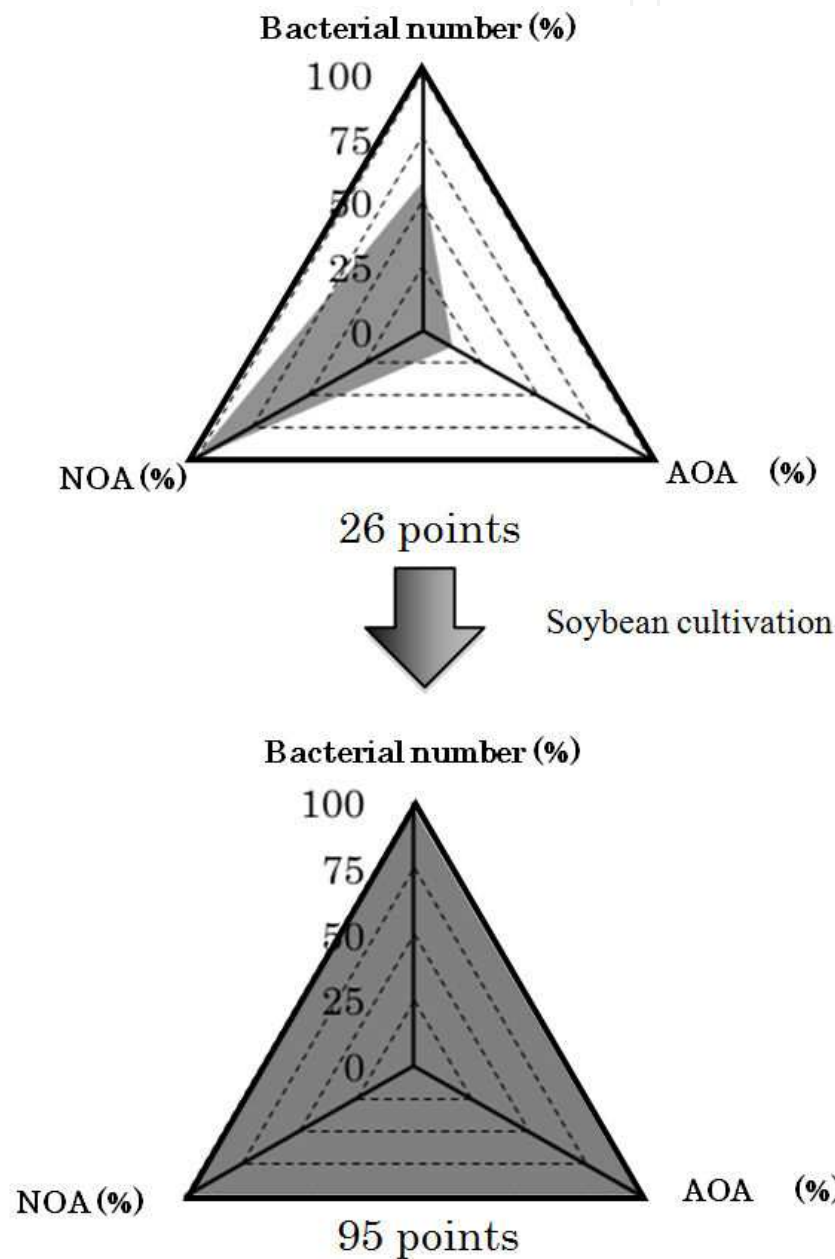


Figure 6. Effect of soybean cultivation on nitrogen circulation activity in soil

Component	Value
Total carbon	450,000 mg/kg
Total nitrogen	87,500 mg/kg
Total phosphorous	6,100 mg/kg
Total potassium	18,900 mg/kg
C/N ratio	5.1

Table 1. Components of soybean meal

4. Bioactive peptides from soybean protein

4.1. Plant growth promoting peptides from soybean waste

For efficient use of soybean waste, it is treated with an alkaline protease from *Bacillus circulans* HA12 (degraded soybean meal products; DSP) [4, 6]. Plant growth promotion by DSP has been investigated using various plant species [30]. The fresh weight of *Brassica rapa* was shown to be increased by 25% through the addition of DSP (12 mg-peptides/kg-soil) (Figure 7). The growth of *Solanum tuberosum* L., *Solanum lycopersicum*, and *Brassica juncea* were also promoted by addition of DSP. Moreover, DSP also produced thicker roots than a chemical fertilizer, indicating that DSP contains bioactive peptides for plant growth.



Figure 7. Plant growth-promoting effect of DSP, A: Chemical fertilizer, B; DSP.

4.2. Root hair promoting peptide in DSP

The number of root hairs in *B. rapa* was increased and each was elongated when DSP (30 µg/ml) was added (Figure 8) to the soil. In order to analyze the root hair promoting effect by DSP, the structure of the root hair promoting peptide (RHPP) in DSP was investigated [6]. Degraded products of Kunitz trypsin inhibitor (KTI) in soybean protein showed higher root hair promoting activity, and the RHPP was purified by several chromatographic steps from degraded products of KTI.

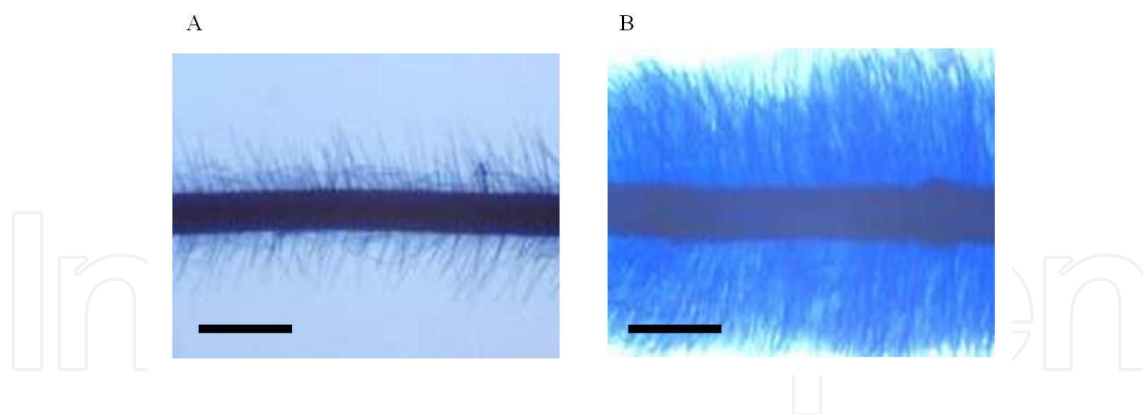


Figure 8. Root hair promoting effect of DSP, A: Root of *Brassica rapa* grown in plant growth medium, B: root of *B. rapa* grown with DSP in plant growth medium. Bar denotes 1 mm.

The molecular mass of RHPP was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [6]. The molecular weight of the bioactive peptide was 1,198.2 Da (Figure 9), and the molecular weight of the amino acid sequence in KTI was searched. Positions 27–38 in KTI (Gly-Gly-Ile-Arg-Ala-Ala-Pro-Thr-Gly-Asn-Glu-Arg) were identical to this molecular weight, and this peptide was thus designated as the RHPP (Figure 9). The RHPP that was chemically synthesized was also shown to have root hair promoting activity (data not shown).

¹DFVLDNEGNPLENGGTYIILSDITAFGGIRAAPTGNERCP⁴⁰
⁴¹LTVVQSRNELDKGIGTIISPSYRIRFIAEGHPLSLKFDSF⁸⁰
⁸¹AVIMLCVGIPTEWSVVEDLPEGPAVKIGENKDAMDGWFR¹²⁰
¹²¹ERVSDDEFNNYKLVFCPQQREDDKCGDIGISIDDDGHTR¹⁶⁰
¹⁶¹LVVSKNKPLVVQFQKLDKESL¹⁸¹

Figure 9. Amino acid sequence of RHPP in Kunitz trypsin inhibitor, the RHPP amino acid sequence is shown by gray box.

5. Novel plant bioactive peptides from other legume

Many other legumes form root-nodules with nitrogen fixing bacteria. The nitrogen fixing bacteria related to legume cultivation are classified into 13 genera (*Rhizobium*, *Ensifer*, *Mesorhizobium*, *Bradyrhizobium*, *Methylobacterium*, *Azorhizobium*, *Devosia*, *Burkholderia*, *Phyllobacterium*, *Microvirga*, *Ochrobactrum*, *Cupriavidus*, and *Shinella*) and 98 species [31].

Legumes such as *Astragalus sinicus*, *Trifolium repens*, and *Arachis hypogaea* are cultivated as green manure for the improvement of soil fertility. The host specificity of the nitrogen fixing bacteria, *M. huakuii*, *R. trifolii*, and *Bradyrhizobium* sp., are very high, infecting *A. sinicus*, *T.*

repens, and *A. hypogaea*, respectively [32]. These legumes are rich in proteins and form root-nodules via the same mechanisms as soybean.

In order to find novel bioactive peptides, attempts to degrade protein biomass from *A. hypogaea* by various proteases (thermolysin, subtilisin, proteinaseK, and trypsin) were made. Bio-activities of root hair and lateral root formation were found by degradation with proteinaseK (Figure 10). Degraded products of *A. hypogaea* by proteinase K (30 µg/ml) showed strong root hair promoting activity at the same level as DSP. Moreover, degraded products of *A. hypogaea* promoted lateral root growth in *B. rapa*, suggesting that degradation of legume proteins has a possibility to produce new bioactive peptides.

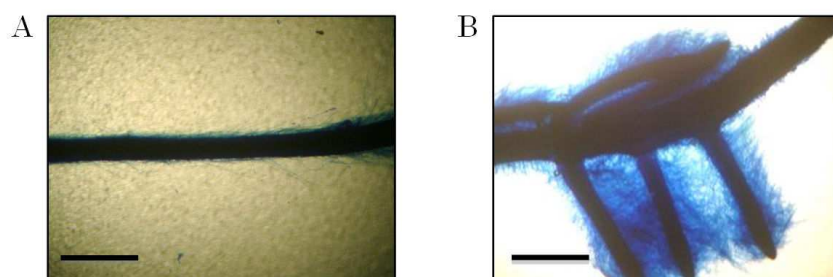


Figure 10. Bioactive effect of degraded products of *A. hypogaea* on root of *B. rapa*, A: Root of *Brassica rapa* grown in plant growth medium, B: root of *B. rapa* grown with degraded products of *A. hypogaea*. Bar denotes 1 mm.

6. Conclusion

Soybean supplies nitrogen into the soil environment by forming root nodules and accumulating protein in its seed. Soybean cultivation has been shown to enhance nitrogen circulation by about 3.6 times accompanied with increases in nitrogen fixing bacteria.

DSP has been shown to increase the fresh weight of plants, and a peptide from DSP promoted root hair formation in *B. rapa*. Moreover, other bioactive peptides were found by degradation of proteins from *A. hypogaea* with proteinaseK treatment. The proteins of legumes will also become nitrogen sources for plant growth and the soil environment.

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