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https://doi.org/10.5109/10066

出版情報：九州大学大学院農学研究院紀要. 53 (1), pp.33-38, 2008-02-28. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **53** (1), 33–38 (2008)

The Difference between the Micronutrients Content of Seedling's Root and Root Hair in Several Plant Species

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(*Received November 8, 2007 and accepted November 30, 2007*)

It was reported in soybean that the content of Fe and Co microelements of the root hair invaded by rhizobium during the process of nodule formation was higher than that of the root. To confirm this point, a supplementary experiment was carried out using several applicable plants, soybeans, lupine, pea, corn and pumpkin. Root hair was separated in liquid nitrogen from the roots of those seedlings. The separated root hair of 20 mg, or the residual root of 200 mg was digested in a microwave wet digestion device of closed system by using hydrogen peroxide and nitric acid. After wet–digestion, the content of micronutrients (Mn, Fe, Co, Ni, Cu and Zn) was measured by using a furnace atomic absorption spectrometer. This result indicates that micronutrients except for Co could be measured in a small amount of about 20 mg.

Fe content was higher in root hair than in root irrespective of a monocotyledonous, a dicotyledonous, a leguminous or a non–leguminous plant. In seedlings, it became clear that most of the Fe storage in seed was accumulated in the root hair. Mo was similarly accumulated in root hair and root of soybeans and pumpkin. In corn, Mo content in root hair was low in comparison with that of root. In other words, the Mo content in root hair of dicotyledonous plants was higher than that of monocotyledonous plants. There was no great difference in the content of Mn, Zn and Cu between root and root hair and among plant species.

From these results, it appeared that the micronutrients stocked in seed of leguminous plants might be transferred to the root hair. Moreover, the accumulation of some peculiar element (Ca, Co and Fe) known in root hair could be guessed to be related to an active absorption from nutrient solution during germination.

INTRODUCTION

A nodule of leguminous plant is the uniquely differentiated new organ so that the nitrogen fixation by rhizobia could function very efficiently (van Rhijn and Vanderleyden, 1995). Nodulation starts from the mutual recognition between rhizobia and a host plant. The rhizobia induce curling of host root hair and entry into the plant root through an infection thread which originate in a deformed root hair. Subsequently, the cortical cells of root of leguminous plant are made to differentiate, and it is considered to form the new organ, nodule (Calvert *et al*., 1984; Newcomb *et al*., 1979; Wood and Newcomb, 1989). Instead the photosynthetates depending on the host plant as the energy source in the nodule, rhizobia (bacteroids) can fix N_2 to produce nitrogenous compounds that are necessary for the growth of the host plants and supply them (Brewin, 1991; Kijne, 1992). Various inorganic elements are also necessary with carbon and nitrogen so that the rhizobia may invade into root hair, multiply, form the nodule and express the ability to fix N_2. The requirement for mineral nutrients of rhizobia and nodule is thought to differ from that of host leguminous plant (Werner and Kuhlmann, 1985; Bhanavase and Pstil, 1993). Therefore, the condition of inorganic nutrition of the leguminous plant can be thought to influence not only the growth of host plant but also nodule formation and N_2 fixation.

Until now, the following research was carried out about the influence of inorganic nutrients on nodulation and N_2 fixation. Sharma and Jauhari (1970) reported that the foliar application of 0.1% ammonium molybdate solution at 40 days after sowing increased the nodule number of pea plant. In pot experiment with Delhi soil (pH 7.2), Iswaram *et al*. (1972) reported an increased nitrogen content in pea plant, when it is inoculated with rhizobia cultured with a medium containing Mo. Hashimoto and Yamasaki (1976) showed that nodules of soybean were unusually enlarged on Mo application. Moreover, Robson *et al*. (1979) showed that when lupinus (*Lupinus angustifolius* L.) was grown for 11 weeks in soil applied with 9 mg Co per pot, the yield and nitrogen content increased, and the number of bacteroids increased in the nodules. Yadav and Shukla (1983) reported that in the pot experiment with chick pea (*Cicer arietinum* L.), when the dose of Zn increase to 10 μg g^{-1} soil, the plant dry weight increased and furthermore when the dose of Zn increase to 7.5 μg g^{-1} soil, the ability of N_2 fixation increased. Tang *et al*. (1990) showed that Fe deficiency influenced the nodulation more strongly than the growth of the plant, and large amount of Fe should be more necessary for the establishment of the symbiosis between rhizobia and host plant than for their growth. Yamagishi and Yamamoto (1994) stated that when a soybean was hydroponically cultured with B deficient nutrient solution, the seed

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yield was decreased. Also, they clarified that the B concentration of nutrient solution to get the highest seed yield and nodule formation was 15 to 180 μg B L^{-1} (1.4 to 16 μM), the ability of N_2 fixation was promoted and the nodule weight was increased with 29 to 88 μg B L^{-1}. As mentioned above, there are many cases that the moderate application of micronutrients makes to promote the plant growth, the nodulation and the N_2 fixation.

Recently, Werner and Kuhlmann (1985) showed that the Ca, Co and Fe contents of the root hair of soybean seedlings were especially high in comparison with those of root, and also higher than those of the root hair and root of wheat seedlings as a result of an analysis by the PIXE (Proton Induced X–ray Emission) method (Kuhlmann *et al*., 1982). From this result and the fact that root hair is a first target when rhizobia infect the root of leguminous plant, it could be thought that rhizobial requirement for the elements such as Ca, Co and Fe is especially high. However, from these results, it is difficult to conclude at once that the inorganic element composition of the soybean root hair has peculiarity.

In this study, we made a hypothesis that rhizobia required the peculiar inorganic nutrient before they adhered on and invaded to root hair of leguminous plant, and their nodules were formed on the root. Therefore, pumpkin and corn; dicotyledonous and monocotyledonous plant respectively, which don't form the nodule by the infection of rhizobia; soybean, pea and lupine, which are leguminous and dicotyledonous plant, were used as plant material. The content of inorganic nutrient was compared between root hair and root of each plant and among plant species. And, the conditions for measurement by a furnace atomic absorption spectrometry were examined using a small amount of sample (root hair, root).

MATERIALS AND METHOD

Plant materials

Soybean (*Glycine max* L. Merr.) cultivars CNS, Hill and Orihime, lupine (*Lupinus luteus* L.) cv. Yellow lupine, pea (*Pisum sativum* L.) cv. Hakuryu, corn (*Zea mays* L) cv. Golden Cross Bantam and pumpkin (*Cucurbita moschata* Duch.) cv. Shintosa No. 1 were used as plant materials in our present study. Soybean, lupine, pea are leguminous plants. Corn (monocotyledonous) plant and pumpkin (dicotyledonous) are non–legumenous plants.

Seed sowing and germination

The culture methods for the effective occurance of root hair described on our previous report (Yamakawa and Taira, 2005) were used basically. In other words, 1 mM $CaSO_4$ solution adjusted to pH 6 with 1 mM $Ca(OH)_2$ solution served as a culture solution and the solution autoclaved for 20 min at 121 ˚C was used as the sterilized culture solution. Petri dishes were used for germination beds in which two sheets of No. 2 filter papers and one sheet of No. 4A filter paper (Advantec Toyo, Tokyo, Japan) were lapped on the body of petri dish (diameter 150 mm), and one sheet of No. 2 filter paper was spread inside the cover plate. The filter papers were wetted by the culture solution, and the beds were wrapped with the aluminum foil, and sterilized for 20 min at 121 ˚C.

a) Soybean and lupine: Seeds were sterilized by immersion in a sodium hypochlorite solution (25 g kg^{-1} as active chlorine) for 5 min, washed five times with 99% ethanol, and rinsed five to six times in sterile deionized water to remove ethanol lefted on the seed surface. An aliquot of the sterilized culture solution was added into the sterilized germination bed to wet the filter papers in it, and the extra culture solution was discarded by decantation. Fifty seeds were sown in a bed and germinated for 3 days at 25 ˚C, 60% relative humidity in a growth cabinet (MLR–350H, SANYO, Tokyo, Japan) under dark condition. Fifteen milliliters of the sterilized culture solution were applied on seeds at 24 hours after sowing. All materials used for seed sowing and cultivation were sterilized, and these operations were also carried out under an aseptic condition.

b) Corn: Because the surface of seed was coated with Benomyl [Methyl 1–(butylcarbamoyl)–2–benzimidazolecarbamate], seeds were washed 5 to 6 times using the sterilized culture solution to take out the germicide. And these seeds were sown and grown in the same method as *a) Soybean and lupine*.

c) Pumpkin: Because the surface of seed was coated with Captan [N–(trichloromethylthio) cyclohex–4–ene–1,2–dicaboximid], seeds were washed, sown and grown in the same method as *b) Corn* except that they were germinated for 4 days.

d) Pea: Seeds was sown and grown in the same method as *a) Soybean and lupine* except that 35 seeds per bed were sown and germinated for 5 days.

Root hair separation

The separation of root hair followed the method of Röhm and Werner (1986) fundamentally. A series of operation from the germination to the root hair separation was carried out twice in all plant species. The roots of seedlings were cut at the base of the radicle, dropped into a beaker containing liquid nitrogen, and stirred for five minutes in the first operation or for ten minutes in the second one. For the individual separation, 200 to 250 seedlings were used. Because the root hairs were floated in liquid nitrogen, they were stripped off the roots which settled in the bottom of the beaker. The root hairs were then collected by decanting the liquid nitrogen fraction into another beaker after stirring at once. Next, an aliquot of liquid nitrogen was added to the beaker that a part of the root hair and the root settled, and the stirring and decantation was repeated three more times. The root hair and the root were freeze–dried after preservation at –80 ˚C, and the dry weight was measured.

Microelement analysis of the root hair and the root

The content of microelements (Zn, Mn, Cu, Fe, Ni, Co and Mo) of the root hair and the root was measured by a furnace atomic absorption spectrometer (SIMMA6000, Perkin–Elmer, Norwalk, USA). At the same time, the micronutrient content of a NIES (National Institute for Environmental Studies) environment standard sample No. 1, pepperbush (Okamoto, 1980) was analyzed. NIES environment standard sample No. 1 was used as the guarantee value and the measurement performed using the at least four independent analyzing methods, an atomic absorption spectrometry, a flame emission spectrometry, an inductively coupled plasma atomic emission spectrometry, a X–ray fluorescence analysis, an isotope dilution mass spectrometry, an instrumental neutron activation analysis and a radiochemical neutron activation analysis, etc. The analytical precision of this experiment was examined from the result of the standard sample.

Decomposition under wet condition: Two types of decomposition were done, one for Zn, Cu, Fe and Ni, and another for Mo and Co. The root hair and the root were decomposed at two replicate at each root hair separation, corresponding to 4 replicate per each plant species. Pepperbush was decomposed at three replicates. A sample of 200 mg from root and pepperbush and a 20 mg sample from root hair were took into the inner container of the microwave wet digestion device of closed system (MCS950, PRORABO, Paris, France). Concentrated nitric acid (Special grade for heavy metal analysis, Nacalai Tesque, Tokyo, Japan) of 2 mL and 30% hydrogen peroxide (ultrapur grade, Cica–MERCK, Tokyo, Japan) of 1 mL were added into that container. It was stood for more than ten minutes until these reagents permeated into the sample. The inner container was delivered to the outer container, and set on the decomposition container rack. The decomposition was carried out in accordance with the program (All process: 30 min) that was recommended with this device. That was left until the internal pressure of the inner container felt down to ambient pressure.

The dilution of decomposed solution: About 3 mL of decomposed solution for analysis of Zn, Mn, Cu, Fe and Ni was decanted into 100–mL plastic volumetric flask and filled up with a super–pure water. For analysis of Co and Mo, about 3 mL of decomposed solution was mixed with 3 mL of super–pure water and the volume of the mixture estimated by weighting. The dilution was in accordance with the dilution magnification of Table 1 and kept in 100–mL polyethylene bottles. These dilution magnifications were decided as the measured value kept within the range of a standard curve by SIMMA6000. These 100–mL polyethylene flasks and 100–mL polyethylene bottles were soaked in 1:1 hydrochloric acid for more than one night, and washed with a deionized water and a super–pure water just before using.

Table 1. The dilution magnification of the original digestion solution of each sample

Sample	Fe	Mn	Zn	Cu	Ni	Mo	Co
Root	100	10	40	10	10	1	1
Root hair	40	1	4	1	1	1	1
Pepperbush	200	400	200	10	10	4	200

Standard solution: As a standard solution for the measurement of Zn, Mn, Cu, Ni and Co, ICP Multi–element standard solution VI (MERCK, Darmstadt, Germany) was used. For measurement of Fe, GFAAS mixed standard solution (Perkin–Elmer, Norwalk, USA) was used. For Mo, Molybdenum standard solution (Cica–MERCK, Tokyo, Japan) was used. The range of concentration of the standard solution was made referring to a measurement detection limit of SIMMA6000, that is 0–5 ppb as for Zn, 0–10 ppb as for Mn, Cu, Fe and Ni, and 0–1 ppb as for Co and Mo.

Condition of measurement: Referring to the recommended condition for measurement of SIMMA6000, the incineration temperature for each of Zn, Mn, Cu, Fe and Ni elements was set up 300 ˚C lower than that of measurement recommendation, and that of Co and Mo was set up at 1100 ˚C to measure two elements at the same time. The temperature of measurement conditions was set up in accordance to the recommended conditions except for incineration (Table 2).

Measurement process: One milliliter of the diluted solution was added to a sample cup. A sample volume of 20 μL, replicated 3 times was set up for in an auto–sampler. The sample cup was immersed in 2% of nitric acid and washed with super–pure water just before using.

Table 2. Recommended condition of measurement for furnace atomic absorption spectrometry analysis

	Fe	Mn	Zn	Cu	Ni	Mo	Co
Incineration Temperature (˚C)	1400	1300	700	1200	1100	1500	1400
Atomization Temperature (˚C)	2100	1900	1800	1900	2300	2400	2400
Atomization time (s)	3	3	3	5	5	5	4

RESULTS AND DISCCUTION

The amount and quality of separated root hair

The dry weight of the root hair and the root collected after the two times separation was shown in Table 3. The root weight per individual was the large values 5.5 to 6.7 mg in soybean and 6.0 to 6.7 mg in lupine, the middle values 3.6 to 4.6 mg in pea and 3.0 to 3.7 mg in pumpkin, and the small values 2.7 to 2.8 mg in corn. This difference could be thought to reflect the difference between dicotyledonous and monocotyledonous plant and in seed size. The root hair weight per individual was different between the two separations for 5 min and 10 min. The second separation for 10 min showed a higher weigh of root hair than the first try for 5 min except for soybean cv. CNS. As for this cause, it could be thought that the root hair was completely separated from the root surface or that small piece of root tip was mixed in beaker during root hair collection in case of decantation of liquid nitrogen. Therefore, the existence of the root hair on root surface at the second separation was confirmed with an optical microscope

Table 3. Harvesting amount of the root and the root hair from each plant species

Species	Cultivar	Frequency	Germinated seed	Germination rate (%)	Root		Root hair	
					g	mg plant^{-1}	mg	μg plant^{-1}
Soybean	CNS	1	870	96.6	5.8	6.7	240	276
		2	866	96.2	5.3	6.2	226	261
	Hill	1	867	96.3	5.0	5.8	87	100
		2	876	97.3	5.4	6.1	125	143
	Orihime	1	890	98.8	4.9	5.5	230	258
		2	882	98.0	5.4	6.1	326	370
Pea	Hakuryu	1	853	93.7	3.1	3.6	179	210
		2	483	92.0	2.4	4.9	136	282
Lupine	Yellow lupine	1	796	88.4	5.4	6.7	188	236
		2	803	89.2	4.8	6.0	214	266
Pumpkin	Shintosa No. 1	1	844	93.7	2.6	3.0	243	288
		2	237	94.8	0.9	3.7	73	308
Corn	GCB	1	870	96.6	2.4	2.7	133	153
		2	435	96.6	1.2	2.8	70	162

Difference between two replicates was only stirring time (The first time: five minutes and the second time: ten minutes) in the case of root hair separation, and the conditions of cultivation and separation were the same. GCB in column of species shows the abbreviation of Golden Cross Bantam.

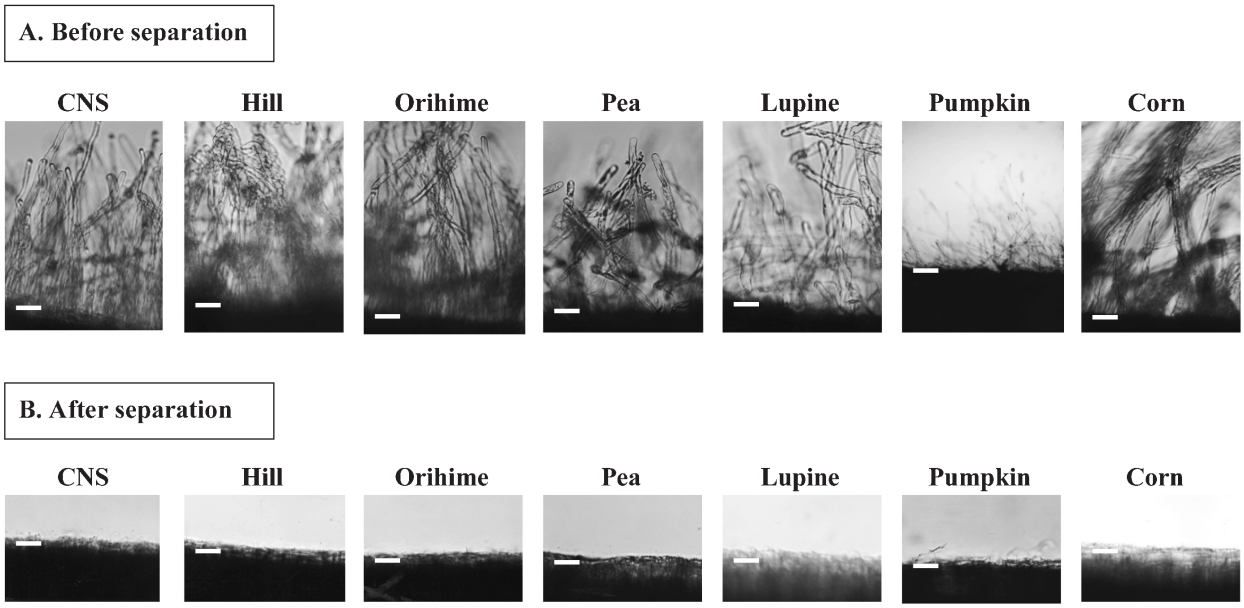

Photo. 1. The occurrence conditions of the root hair before the root hair separation and the root surface conditions after the root hair separation.
The length of each white bar in the photograph shows 100 μm, and that position means the surface of the root.

(OPTIPHOT, Nikon, Tokyo, Japan). That result showed that the root hair was completely removed from the root surface (Photo. 1). But, when the collected root hair was floated on deionized water and observed with the optical microscope, a small amount of root tip tissue was mixed in the root hair in the pumpkin (data not shown). The separation of the root hair from the roots was concluded to carry out completely except for the pumpkin. Because the roots of pumpkin were thin in comparison with the roots of the other species, it was guessed that some piece of root tip was mixed together in beaker for the root hair collection in case of decantation. Moreover, the especially large difference between two separations was also seen in the weight of root hair per individual with soybean cultivars Hill and Orhihime, and pea plant. This result was thought to be related to the water environment inside the petri dishes at the emergence stage of root hair. Indeed, after the filter papers were wet with a sterilized culture solution at the sowing time and that an extra culture solution was removed from beds, the amount of culture solution remained in the petri dishes was delicately different in every bed. Although, the optimum water condition for the occurrence of root hair was examined fully in the previous paper (Yamakawa and Taira, 2005), the effect of seed size was examined insufficiently. Therefore, the number of seeds sown per a petri dish may be adjusted in accordance with the seed size for a best occurrence of root hair emergency.

The precision of this analysis

A result of measurement of pepperbush as the average of 3 replicates was shown in Table 4. This result shows that some conditions modified from that of recommendation for the analysis by SIMMA6000 brought about no problem in the reliability of result measured with this analysis method.

Table 4. Measurement value and guarantee value of NIES environment standard sample No. 1, Pepperbush

Microelement	Measurement value	Guarantee value
	(μg g DW^{-1})	
Mn	1797 ± 25	2030 ± 170
Zn	366 ± 3.6	340 ± 20
Fe	244 ± 14	205 ± 17
Co	22 ± 1.6	23 ± 3.0
Cu	14 ± 0.7	12 ± 1.0
Ni	9 ± 0.5	9 ± 0.6
Mo	1 ± 0.1	–

All measurement value shows mean ± S. D. of three replicates. Guarantee value referred to Okamoto (1980).

The content of microelements of the root and the root hair

The concentration of microelements contained in the root and root hair of each plant species was shown in Table 5. No difference was seen in the analytical data of each sample collected by the root hair separation of two times. From these results, no difference among plant species was observed in the concentration of Zn, Mn, Cu and Ni between the root and the root hair. The concentration of Co was very low in both root and root hair. It was a measurement value around a detection limit with a high measurement error for all samples. Therefore, it became clear that using a small amount of sample of about 20 mg, micronutrients (Zn, Mn, Cu, Fe, Ni and Mo) except for Co could be analyzed by the combination of a furnace atomic absorption spectrometry and a microwave wet–digestion of closed system.

The concentration of Fe was higher about 2 to 15 times in the root hair than that in the root of all plant species used in this experiment with no inoculation of rhizobium irrespective of a monocotyledonous, a dicotyledonous, a leguminous or a non–leguminous plant. In seedlings, it became clear that most of the Fe storage in seed was accumulated in the root hair. Therefore, this high concentration of Fe in root hair was thought to be related to the root hair development but not to the adhesion and the infection of rhizobia to root hair.

The concentration of Mo was remarkably low in the root hair of corn, which is a monocotyledonous plant, incapable of forming nodule. In contrast, it was high in the root and the root hair of soybean, a dicotyledonous plant forming nodules, and also in the root hair of lupine and pea, which can also form nodules. In other words, the Mo content in root hair of dicotyledonous plants was higher than that of monocotyledonous plants. However, the concentration of Mo in the root and root hair of pumpkin which can't form nodules was also high. Furthermore, Mo content in root hair was low in comparison with that of root. Therefore, it could be guessed that the possibility of the direct relation between the high concentration of Mo of the root hair and the nodulation was low.

Indeed, as the germination was carried out without supplying nutrients except for Ca as nutrient solution, this result indicate that the inorganic elements existing in the seed are distributed to in the root hair during germination. This result is in agreement with Werner and Kuhlman (1985) who supported that the accumulation of Ca, Fe and Co in root hair of leguminous plants was related to an active absorption from the rhizophere during the germination. However, the analysis value of each microelement in this study is lower than that by Werner and Kuhlman (1985). Therefore the difference in the analysis methods and the conditions of germination influence these results.

Table 5. The concentration of each microelement contained in the root and the root hair of each plant species

Species	Cultivar	Position	Fe	Mn	Zn	Cu	Ni	Mo	Co
			μg g DW^{-1}					ng g DW^{-1}	
Soybean	CNS	Root	65 ±11	12 ± 0	60 ± 1	11 ± 0	11 ± 0	858 ± 53	–
		Root hair	738 ± 97	14 ± 1	65 ± 2	12 ± 2	10 ± 1	808 ± 98	78 ± 39
	Hill	Root	56 ± 6	9 ± 0	61 ± 4	9 ± 0	7 ± 0	570 ± 21	–
		Root hair	548 ± 38	15 ± 2	92 ± 5	10 ± 5	10 ± 1	393 ± 22	32 ± 2
	Orihime	Root	61 ± 6	7 ± 0	59 ± 4	13 ± 1	10 ± 1	716 ± 135	–
		Root hair	511 ± 40	8 ± 0	69 ± 10	13 ± 1	10 ± 2	861 ± 4	65 ± 28
Pea	Hakuryu	Root	131 ± 12	5 ± 0	56 ± 3	19 ± 2	8 ± 0	697 ± 23	–
		Root hair	519 ± 39	6 ± 0	61 ± 6	22 ± 1	6 ± 1	–	–
Lupine	Yellow lupine	Root	99 ± 30	13 ± 2	82 ± 8	18 ± 0	7 ± 0	335 ± 11	–
		Root hair	146 ± 64	24 ± 1	119 ± 15	20 ± 0	6 ± 0	119 ± 60	–
Pumpkin	Shintosa No. 1	Root	74 ± 9	14 ± 0	68 ± 7	17 ± 1	31 ± 3	416 ± 44	–
		Root hair	573 ± 127	15 ± 1	92 ± 19	13 ± 1	27 ± 1	400 ± 35	40 ± 44
Corn	GCB	Root	44 ± 6	10 ± 1	92 ± 6	9 ± 0	7 ± 2	–	–
		Root hair	663 ± 30	10 ± 0	72 ± 8	8 ± 2	14 ± 1	50 ± 16	32 ± 19

All data shows mean ± S. D. of four replicates, and – shows less than a detection limit. GCB in column of species shows the abbreviation of Golden Cross Bantam.

As a future research, it will be of big interest to investigate about how a component difference in micronutrients of the culture medium would influence to nodulation effect of cultivated leguminous plant. More research should also be addressed about the microelements promoting or repressing the nodulation.

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