THE INFLUENCE OF PLANT HORMONES ON THE FORMATION OF INFECTION THREAD INTO ROOT HAIR OF TRIFOLIUM REPENS L. BY RHIZOBIUM TRIFOLII K102

THE INFLUENCE OF PLANT HORMONES ON THE FORMATION OF INFECTION THREAD INTO ROOT HAIR OF *TRIFOLIUM REPENS L.* BY *RHIZOBIUM TRIFOLII K102*

By

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Abstract

To analyse the mechanism of infection thread formation into the root hair of white clover, we examined on the influence of plant hormone of five kinds, indole-3-acetic acid, α-naphthalene acetic acid, 2,4-dichlorophenoxy acetic acid, kinetin and gibberellin, employing clover excised root tip method. The excised root tip was cultured on the situation which was inserted in capillary tube, therein containing with nutrient solution and plant hormone. In the case of doses of higher concentration of IAA as well as NAA, the number of infection thread increased and conversely its number exhibited tendency of decrease gradually by the influence of higher concentration of 2,4-D, kinetin and gibberellin. This phenomenon suggests probably the presence of infectious regulatory system for invasion of rhizobia in root of leguminous plant, which is disorganised only by doses of higher concentration of IAA or NAA. It was also proved that there is no correlation between the formation of infection thread and cell division of the host plant, in the present investigation.

Introduction

It is well known that the host specificity of rhizobial species is one strict character, as utilizing for the classification of *Rhizobiaceae*, and it is also obviously that the formation of infection thread in the root hair of leguminous plants is observed only in the host, which is corresponding to the microorganisms.

It is considered probably that the analysis of infectious pathway into root of leguminous plant by rhizobial species can be applied to the following approachable three ways. The first way is the transformation, examined from bacterial genetics which is mainly investigated by Balassa et al. (1, 2, 3). The second way is the relationship between nodule formation and the components of its cultured nutrients, which was investigated by the method of excised root tip culture by Raggio et al. (4). The third approach is the analysis of substances on mechanism of infectious pathway as

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especially concerning to the role of polysaccharide, which was demonstrated by Nutman and Ljunggren (5, 6). The central problem of this work is to elucidate the primary stage of infection by *Rhizobium* into host plant along the second way among those three approaches. Raggio and Raggio and Torrey (4) reported that excised roots of *Glycine max* and *Phaseolus vulgaris*, inoculated with their homologous *Rhizobium* strains, developed nodules. Valera and Alexander (7) demonstrated that nodulation of excised roots of *Medicago sativa* was enhanced by an extract of alfalfa seeds but not by several other substances and coconut water exerted a similar influence upon the formation of nodules on excised roots of *Glycine max* and *Phaseolus vulgaris*. However, the biochemical and microscopical details of pathway of the infectious mechanism into the leguminous root hair have been still unsolved.

The present investigation was observed on the relationship between infection thread formation and plant hormones at the primary stage of infection by the complex methods of Fåhraeus (8) and Raggio (4) with some modification.

Materials and Methods

Bacterial strain and culture medium.

The test organism mainly used in this studies was *Rhizobium trifolii K102* (9). Using other Rhizobium species, *R. leguminosarum*, *R. meliloti* and *R. lupini*, were isolated from each their host plant root nodule. The constituent of the liquid medium (YM-medium) was described previously by Keele et al. (10). The organisms were cultured in YM-medium at 28°C for overnight by shaker. These organisms were harvested by centrifugation at 9000 rpm for 30 min and suspended in sterilized water as used bacterial titer of 10^7/ml.

Cultivation of root tips and measurement of infection thread number.

Root of white clover (*Trifolium repens L.*) was cultured essentially by the technique outlined by Fåhraeus (8). The seeds were sterilized by the mixing solution of 0.2% formalin, 90% alcohol and 0.1% $HgCl_2$: (1:1:1 by volume), for 1 min, followed several rinses in sterilized water. Sterilized seeds were transferred to petri-dishes of 9 cm diameter, in which sterilized water was added about 5 ml. The seeds were cultured for 2 days at 25°C under condition of illuminating fluorescent lamp all day.

Two days after germination, clover root tips were excised 8 mm in length, the base of excised root tip 1 mm in length was immediately inserted in a capillary tube for microhematocrit determination (diameter of 1.3～1.5 mm and 35 mm in length). Each capillary tube was filled up with basal organic liquid medium as show following composition: nicotinic acid, 0.5 mg; pyridoxine, 0.1 mg; thiamine-HCl, 0.1 mg as mineral nutrient, and several kinds and amounts of carbon source, herein added to plant hormone and distilled water, 1 liter. For selection of most effective carbon source on the infection thread formation, six kinds of sugar, glucose, sucrose, xylose, maltose,

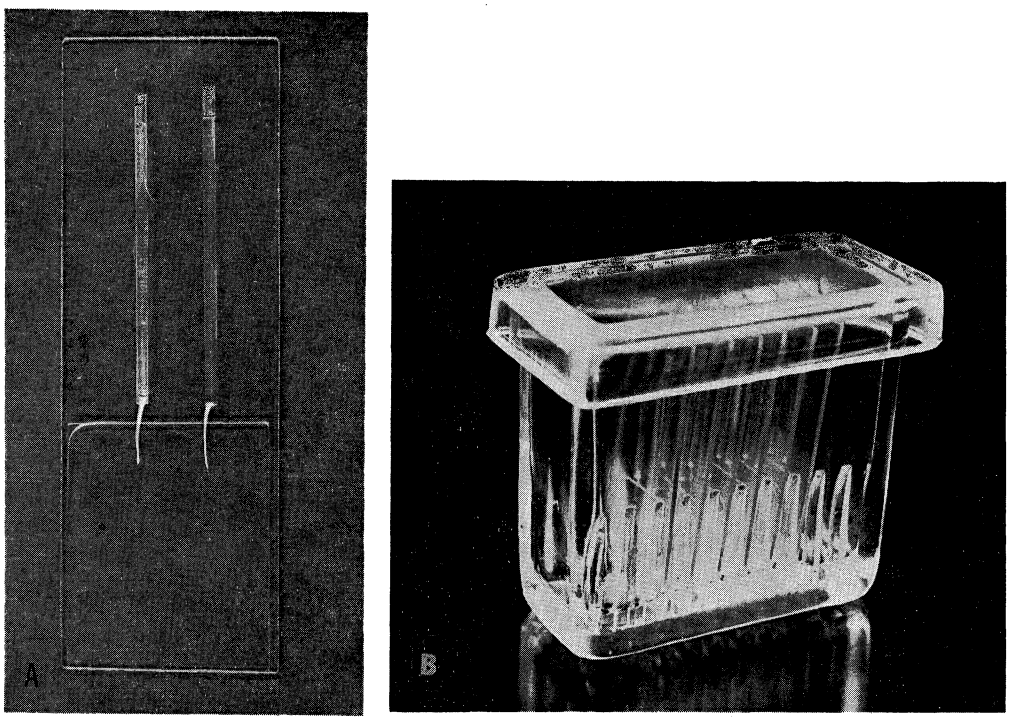

Fig. 1. Experiments with excised root tip growth and infection thread formation in its root hairs by slide glass cultivating method.

A. Capillary tubes are 1.3~1.5×35 mm in size and are filled with organic medium. The tubes are plugged by 2% agar among 4 mm in length from basal end of a tube. Each 8 mm in length of excised root tips are inserted from section of root to 1 mm in length in agar plug. Root inserted tubes are fixed by 2% agar. Cover glass is placed on 0.2 ml of Fåhraeus inorganic solid medium containing within 0.05 ml of *Rhizobium trifolii K102* suspension (cell titer 10^7/ml) and from root tip to the portion of 5 mm in length.

B. The setting slide glass is placed in staining glass chamber for morphology, wherein 10 ml of Fåhraeus liquid medium is poured. This is cultured under fluorescent lamp all day at 25°C for 7 days.

fructose and galactose were examined. Plant hormones were employed several molecules of indole acetic acid (IAA), α-naphthalene acetic acid (NAA), kinetin, 2,4-dichlorophenoxy acetic acid (2,4-D) and gibberellin A3. All nutrient media were sterilized by membrane filter (pore size 0.45 μ).

Inserted root tip in capillary tube was placed on slide glass and fixed with 2% agar at a portion of capillary tube. The settled root tips were inoculated with bacterial cells on the slide glass essentially by the method of Fåhraeus as described in the previous paper (9, 11). Fig. 1 shows the cultivation of root tips on slide glass in staining glass chamber filling up with 10 ml Fåhraeus inorganic liquid medium.

After 7 days, the root hairs were observed carefully under microscope (at magnification of ×200, used Olympus long focus objective lens) and the numbers of infection thread within root hair were counted.

Result

The effect of several carbon sources on the root tip growth and infection thread formation.

To establish the root tip culture of clover, an experiment was examined with the influence of several carbon sources on its growth and infection thread formation into the root hair. The experiment was used the nutrient organic medium, adding 2% several kinds of sugar solution containing within each capillary tubes. The data of the effect of each sugars as carbon source are presented in Table 1. The best carbon source, in which was examined six kinds of carbon source about the root tip growth and infection thread formation, was sucrose as described previously by Raggio et al. (4).

Table 1. Effects of several carbon sources on the growth of excised root tips and infection thread formation.

Sugar	No. of roots used	Mean final length of roots (mm)	No. of infection thread formed roots	Total no. of infection thread (N: nodule)	Mean no. of infection thread per root	Mean no. of infection thread per thread-formed root
cont.	12	8.7	2	3	0.25	1.50
glu.	12	17.1	3	8	0.67	2.67
fru.	12	16.5	3	5 (N 1)	0.42	1.67
suc.	12	18.7	10	44 (N 4)	3.67	4.40
xyl.	14	18.3	1	1	0.07	1.00
mal.	14	19.5	4	4	0.29	1.00
gal.	14	15.7	1	6 (N 2)	0.43	6.00

glu.: glucose, fru.: fructose, suc.: sucrose, xyl.: xylose, mal.: maltose, gal.: galactose.

On the contrary, xylose was most ineffectiveness on the both rate of root tip growth and in the infection thread number per root. Furthermore, other sugars except sucrose were quite poor in the effectiveness on infection thread formation. However, there were no differences between sucrose and other sugars on the growth of excised root.

Table 2. Effects of different concentrations (%) of sucrose on the growth of excised root tips and infection thread formation.

Conc. of Suc. (%)	No. of roots used	Mean final length of roots (mm)	No. of infection thread formed roots	Total no. of infection thread	Mean no. of infection thread per root	Mean no. of infection thread per thread-formed root
0	14	9.4	0	0	0.00	0.00
10	14	17.7	2	14	1.00	7.00
2	12	19.8	10	41	3.42	4.10
0.2	13	11.1	1	7	0.54	7.00
0.02	14	10.5	0	0	0.00	0.00

Table 3. Effects of different concentrations (moles per liter) of five kinds of plant hormone to growth of excised root tips and infection thread formation.

Mole per liter	No. of roots used	Mean final length of roots (mm)	No. of infection thread formed roots	Total no. of infection thread (N: nodule)	Mean no. of infection thread per root	Mean no. of infection thread per thread-formed root
			I A A			
10^{-6}	24	19.4	21	131 (N 1)	5.46	6.23
10^{-5}	24	16.2	24	165 (N 2)	6.88	6.88
10^{-4}	24	12.8	23	212 (N 2)	8.83	9.22
10^{-3}	24	8.4	21	505	21.04	24.04
			N A A			
10^{-6}	12	16.5	10	57	4.75	5.70
10^{-5}	11	14.1	11	58	5.27	5.27
10^{-4}	11	13.3	10	56	5.09	5.60
10^{-3}	12	9.8	12	346	28.50	28.50
			Gibberellin			
10^{-10}	12	19.5	8	19 (N 1)	1.58	3.38
10^{-9}	12	22.5	8	34 (N 1)	2.83	4.25
10^{-8}	12	20.9	7	15	1.25	2.14
10^{-7}	12	19.8	6	9	0.75	1.50
10^{-6}	12	20.4	2	6	0.50	3.00
10^{-5}	10	18.3	3	4	0.40	1.33
10^{-4}	12	19.6	1	5	0.42	5.00
10^{-3}	12	15.8	0	0	0.00	0.00
			2, 4 - D			
10^{-10}	12	13.4	10	47 (N 2)	3.91	4.70
10^{-9}	11	16.8	9	49	4.49	5.44
10^{-8}	12	11.6	0	0	0.00	0.00
10^{-7}	11	14.3	6	10 (N 1)	0.83	1.66
10^{-6}	12	13.1	3	16	1.33	5.33
10^{-5}	12	10.7	10	64	5.33	6.40
10^{-4}	12	9.1	6	20	1.66	3.33
10^{-3}	12	8.1	3	6	0.50	2.00
			Kinetin			
10^{-10}	11	17.5	10	67	6.09	6.70
10^{-9}	10	18.6	8	60	6.00	7.50
10^{-8}	10	21.0	9	52	5.20	5.78
10^{-7}	10	19.6	10	54 (N 1)	5.40	5.40
10^{-6}	12	18.2	9	24	2.00	2.66
10^{-5}	12	12.4	2	7	0.58	3.50
10^{-4}	12	11.4	2	4	0.33	2.00
10^{-3}	12	11.0	0	0	0.00	0.00
			NONE ADDITION OF HORMONE			
cont.	114	18.6	103	494 (N 2)	4.33	4.79

The influence of sucrose concentration.

Table 2 shows that the sucrose amounts containing within capillary tube affected to the growth of root tip and the number of infection thread per root by the 7th day after inoculation. In the cases of the omitted sugar as control and smaller amounts of sucrose (0.02% and 0.2%), they did not affect not only the growth of root tip, but also infection thread number, as compared with 2% sucrose concentration. Thus, the optimum concentration of sucrose was recognized as 2% of cultivating liquid medium from this experiment.

The effect of several plant hormones.

The influences of IAA, NAA, 2,4-D, kinetin and gibberellin on infection thread formation of excised clover roots were also examined. These plant hormones have generally been known as an inhibitor of plant growth and physiological functions in the case of high concentration, especially in the growth of root. Equimolecular amount of IAA and NAA were particularly remarked for their effects on infection thread formation. Table 3 shows the decreasing tendency of root length of clover at the higher concentration of each hormones gradually. On the contrary, there were recognized to rise the number of infection thread with increasing concentration of IAA or NAA, regardless of the decreasing tendency of root length. When 10^{-3}M of NAA was given, the maximum mean number, 28.05 per root tip, of infection thread was obtained, at the root growth was 9.8 mm in length. Similary, the treated plants by IAA of 10^{-3}M also showed mean number of infection thread of 21.04, when the growth of root was 8.4 mm in length. Fig. 2 indicates that the numerous infection threads in the growing region of root which were caused by IAA concentration of 10^{-3}M. In contrast, the other plant hormones, 2,4-D, kinetin and gibberellin, did not affect significantly by any amounts in the number of infection thread. On the basis of those results, it was clarified that the effects of IAA and NAA group, whose plant physiological functions are similary, and of other hormones group in infection thread formation are essentially different.

Discussion

The experimental results were shown that the infection thread number increased only by doses of higher concentration of IAA or NAA (Table 3). From this phenomenon, it is considered that the mechanism of infectious pathway may not be a simplicity. This is also proved from the facts that the number of infection thread was decreased with increasing concentration of 2,4-D, kinetin, and gibberellin gradually and there was not established infection thread formation in the decapitated clover root by used another rhizobial species (*R. lupini*, *R. meliloti*, *R. leguminosarum*), even under the abnormal situation by higher concentration of IAA or NAA. For some infectious stages, the existence of regulatory system, which is disorganised only by higher

concentration of IAA or NAA, is suggested from above observation. Moreover, the existence of other selective device without above regulatory system against uncorresponding rhizobia in their host plants also is supposed. It could not be obtained only by this experiment that a precise answer to the infection thread formation be influenced by any activity of IAA and NAA directly or indirectly.

The seemingly paradoxical situation was observed that, when host cell divisions were inhibited by higher amounts of each hormones, the root hairs were created as well as in normal condition. The infection thread formation was high densely discovered into these newly root hairs at higher amounts of IAA or NAA (Fig. 2). Furthermore, when the host tissue cells were loosed in the cell connections of each other and became callus like state from observing results by microscope, a rise of infection thread number was recognized apparently. From these phenomena, it may be that there is not correlation between cell division and infection thread formation directly. The infection thread probably is formed by activating faculty of infected bacteria. However, we could not definitely conclude whether the constructive materials of infection thread is supplyed from the excised root tissues or infected bacteria themselves.

We must consider to the interaction of leguminous plants and rhizobial species on the primary stage of infection again. It seems following two cases that the infection thread formation is not established by any rhizobial species into unconformable leguminous plants. The first case is that the rhizobia can invade into uncorresponding plant root hair, but wherein their growth is inhibited by some regulatory material containing with host cells and the infection thread therefore can not be formed in this plant. The second case is ensuing that the bacteria are already selected before their invasion has occured at the surface of cell wall of root hair. The above mentions are important points which must be clarified on the primary stage of infectious pathway.

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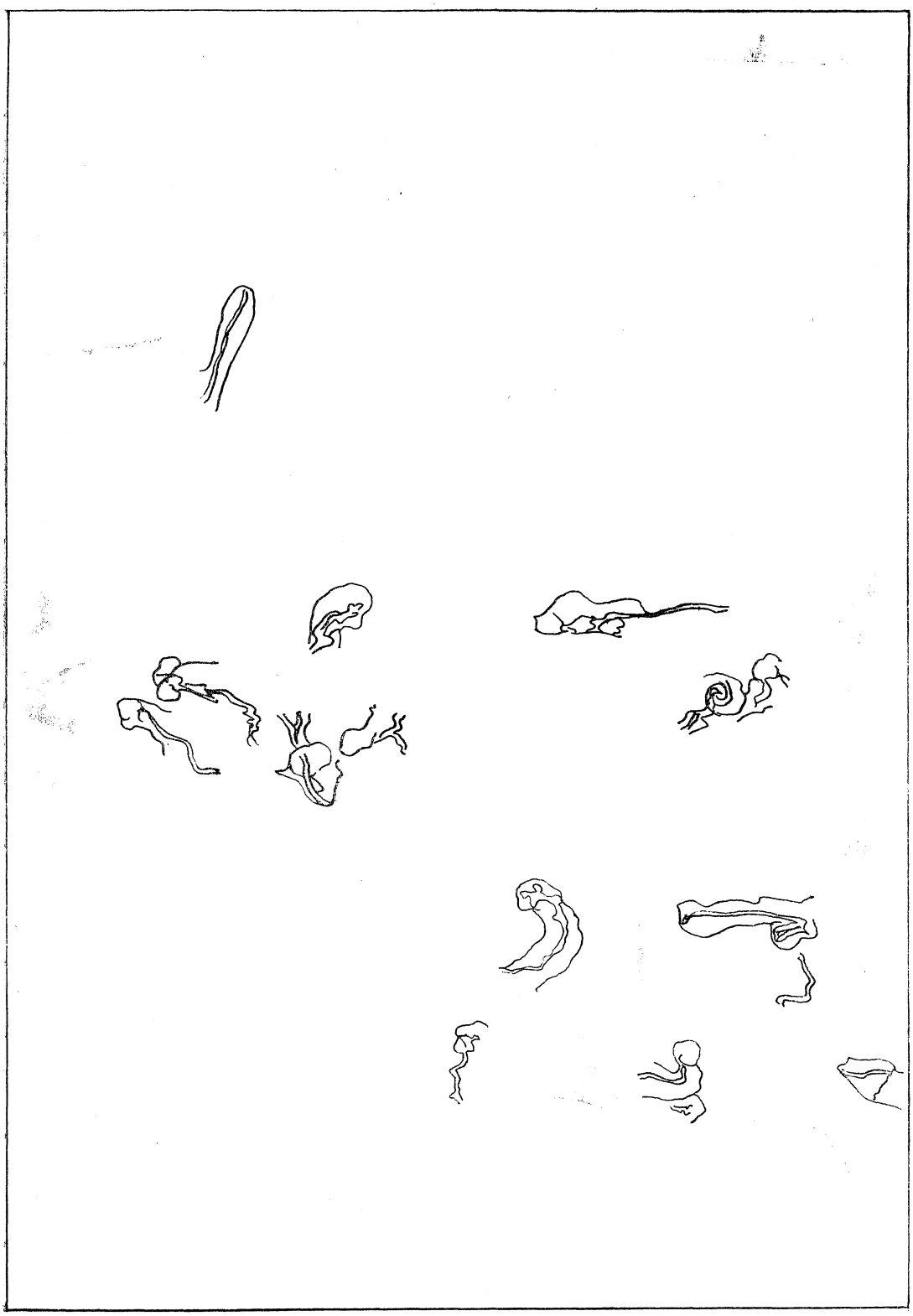

Fig. 2. Microscopical photography of some excised root tip, which was cultured with organic medium containing IAA 10^{-3}M, in the vicinity of growing ponit, 7 days after inoculation.